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(54) Title: TREATMENT FOR VEROTOXIN-PRODUCING ESCHERICHIA COLI

(57) Abstract

The present invention includes methods for generating neutralizing antitoxin directed against verotoxins. In particular, the antitoxin directed against these toxins is produced in avian species using soluble recombinant verotoxin proteins. This avian antitoxin is designed so in the treatment of humans and other animals intoxicated with at least one bacterial toxin, as well as for diagnostic assays to detect the

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TREATMENT FOR VEROTOXIN-PRODUCING ESCHERICHLA COLI

FIELD OF THE INVENTION

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The present invention relates to antitoxin therapy for humans and other animals, and diagnostic assays to detect toxins. Antitoxins which neutralize the pathologic effects of Escherichia coli toxins, such as verotoxin are provided.

BACKGROUND OF THE INVENTION

A. Escherichia coli as a Pathogenic Organism

Escherichia coli is the organism most commonly isolated in clinical microbiology laboratories, as it is usually present as normal flora in the intestines of humans and other animals. However, it is an important cause of intestinal, as well as extraintestinal infections. For example, in a 1984 survey of nosocomial infections in the United States, E. coli was associated with 30.7% of the urinary tract infections, 11.5% of the surgical wound infections, 6.4% of the lower respiratory tract infections, 10.5% of the primary bacteremia cases, 7.0% of the cutaneous infections, and 7.4% of the other infections (J.J. Farmer and M.T. Kelly, "Enterobacteriaceae," in Manual of Clinical Microbiology, Balows et al.(eds), American Society for Microbiology, [1991], p. 365). Surveillance reports from England, Wales and Ireland for 1986 indicate that E. coli was responsible for 5.473 cases of bacteremia (including blood, bone marrow, spleen and heart specimens): of these, 568 were fatal. For spinal fluid specimens, there were 58 cases, with 10 fatalities (J.J. Farmer and M.T. Kelly, "Enterobacteriaceae," in Manual of Clinical Microbiology, Balows et al.(eds), American Society for Microbiology, [1991], p. 366). There are no similar data for United States, as these are not reportable diseases in this country.

Studies in various countries have identified certain serotypes (based on both the O and H antigens) that are associated with the four major groups of *E. coli* recognized as enteric pathogens. Table 1 lists common serotypes included within these groups. The first group includes the classical enteropathogenic serotypes ("EPEC"): the next group includes those that produce heat-labile or heat-stable enterotoxins ("ETEC"): the third group includes the enteroinvasive strains ("EIEC") that mimic *Shigella* strains in their ability to invade and multiply within intestinal epithelial cells: and the fourth group includes strains and serotypes that cause hemorrhagic colitis or produce Shiga-like toxins (or verotoxins) ("VTEC" or "EHEC" [enterohemmorrhagic *E. coli*]).

Table 1.

Pathogenic E. coli Serotypes

Group	Associated Serotypes			
Enterotoxigenic (ETEC)	O6:H16: O8:NM; O8:H9: O11:H27: O15:H11: O20:NM: O25:NM: O25:H42: O27:H7; O27:H20: O63:H12: O78:H11: O78:H12: O85:H7: O114:H21: O115:H21; O126:H9: O128ac:H7; O128ac:H12: O128ac:H21: O148:H28: O149:H4: O159:H4: O159:H20: O166:H27; and O167:H5			
Enteropathogenic (EPEC)	O26:NM; O26:H11; O55:NM; O55:H6: O86:NM: O86:H2; O86:H34; O111ab:NM; O111ab:H2: O111ab:H12: O111ab:H21: O114:H2: O119:H6: O125ac:H21: O127:NM: O127:H6: O127:H9; O127:H21: O128ab:H2: O142:H6; and O158:H23			
Enteroinvasive (EIEC)	O28ac:NM: O29:NM: O112ac:NM: O115:NM: O124:NM: O124:H7: O124:H30: O135:NM: O136:NM: O143:NM: O144:NM: O152:NM: O164:NM: and O167:NM			
Verotoxin-Producing (VTEC))	O1:NM: O2:H5: O2:H7: O4:NM: O4:H10: O5:NM: O5:H16: O6:H1: O18:NM; O18:H7: O25:NM: O26:NM: O26:H11: O26:H32: O38:H21: O39:H4: O45:H2: O50:H7: O55:H7: O55:H10: O82:H8: O84:H2: O91:NM: O91:H21: O103:H2: O111:NM: O111:H8: O111:H30: O111:H34: O113:H7: O113:H21: O114:H48: O115:H10: O1,17:H4: O118:H12: O118:H30: O121:NM: O121:H19: O125:NM: O125:H8; O126:NM: O126:H8: O128:NM: O128:H2: O128:H8: O128:H12: O128:H25: O145:NM: O125:H25: O146:H21: O153:H25: O157:NM: O157:H7: O163:H19: O165:NM: O165:19: and O165:H25			

B. Verotoxin Producing Strains of E. coli

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Although all of these disease-associated serotypes cause potentially life-threatening disease. *E. voli* O157:H7 and other verotoxin-producing strains have recently gained widespread public attention in the United States due to their recently recognized association with two serious extraintestinal diseases, hemolytic uremic syndrome ("HUS") and thrombotic thrombocytopenic purpura ("TTP"). Worldwide, *E. voli* O157:H7 and other verotoxin-producing *E. voli* (VTEC) are an increasingly important human health problem. First identified as a cause of human illness in early 1982 following two outbreaks of food-related hemorrhagic colitis in Oregon and Michigan (M.A. Karmali, "Infection by Verocytotoxin-Producing *Escherichia voli*," Clin. Microbiol. Rev., 2:15-38 [1989]; and L. W. Riley, *et al.* "Hemorrhagic colitis associated with a rare *Escherichia voli* scrotype," New Eng. J. Med.,

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308: 681-685 [1983]), the reported incidence of VTEC-associated disease has risen steadily, with outbreaks occurring in the U.S., Canada, and Europe.

With increased surveillance, *E. coli* O157:H7 has been recognized in other areas of the world including Mexico. China, Argentina, Belgium, and Thailand (N. V. Padhye and M. P. Doyle, "Escherichia coli O157:H7: Epidemiology, pathogenesis and methods for detection in food," J. Food, Prot., 55: 555-565 [1992]; and P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," Epidemiol, Rev., 13: 60 [1991]).

The disease attracted national attention in the U.S. after a major outbreak in the Pacific Northwest that was associated with consumption of undercooked E. coli O157:H7contaminated hamburgers. Over 700 hundred people fell ill (more than 170 were hospitalized) and four young children died (P. Recer, "Experts call for irradiation of meat to protect against food-borne bacteria." Associated Press. 7/12/94 [1994]). Several outbreaks since then have underscored the potential severity and multiple mechanisms for transmission of VTEC-associated diseases (M. Bielaszewská et al., "Verotoxigenic (enterohaemorrhagic) Escherichia coli in infants and toddlers in Czechoslovakia." Infection 18: 352-356 [1990]: A. Caprioli et al.. "Hemolytic-uremic syndrome and Vero cytotoxin-producing Escherichia coli infection in Italy, "J. Infect. Dis., 166: 184-158 [1992]; A. Caprioli, et al., "Community-wide Outbreak of Hemolytic-Uremic Syndrome Associated with Non-O157 Verocytotoxin-Producing Escherichia coli." J. Infect. Dis., 169: 208-211 [1994]; N. Cimolai, "Low frequency of high level Shiga-like toxin production in enteropathogenic Excherichia coli serogroups." Eur. J. Pediatr., 151: 147 [1992]: and R. Voelker., "Panel calls E. coli screening inadequate." Escherichia coli O157:H7--Panel sponsored by the American Gastroenterological. Association Foundation in July 1994. Medical News & Perspectives, J. Amer. Med. Assoc.. 272: 501 [1994]).

While O157:H7 is currently the predominant *E. coli* serotype associated with illness in North America, other serotypes (as shown in Table 1, and in particular O26:H11, O113:H21, O91:H21 and O111:NM) also produce verotoxins which appear to be important in the pathogenesis of gastrointestinal manifestations and the hemolytic uremic syndrome (P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome," Epidemiol. Rev., 13: 60 [1990]; M. M. Levine, *et al.*, "Antibodies to Shiga holotoxin and to two synthetic peptides of the B subunit in sera of patients with *Shigella dysenteriae*.)

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dysentery." J. Clin. Microbiol., 30: 1636-1641 [1992]: and C. R. Dorn. et al., "Properties of Vero cytotoxin producing Escherichia coli of human and animal origin belonging to serotypes other than O157:H7," Epidemiol. Infect., 103: 83-95 [1989]). Since organisms with these serotypes have been shown to cause illness in humans they may assume greater public health importance over time (P. M. Griffin and R. V. Tauxe, "The epidemiology of infections caused by Escherichia coli O157:H7, other enterohemorrhagic E. coli, and the associated hemolytic uremic syndrome," Epidemiol. Rev., 13: 60 [1990]).

Clinicians usually observe cases of hemolytic uremic syndrome ("HUS") clustered in a geographic region. However, small outbreaks are likely to be missed because many laboratories do not routinely screen stool specimens for *E. coli* O157:H7. Many cases related to non-commercial food preparation also probably go unrecognized. Nonetheless, *E. coli* O157:H7 is responsible for a large number of cases, as more than 20,000 cases of *E. coli* O157:H7 infection are reported annually in the U.S., with 400–500 deaths from HUS. However, these estimates were compiled when only 11 states mandated reporting of *E. coli* O157:H7. Twenty-nine states have recently made *E. coli* O157:H7 infection a reportable disease (R. Voelker, "Panel calls *E. coli* screening inadequate: *Escherichia coli* O157:H7: panel sponsored by the American Gastroenterological Association Foundation in July 1994. Medical News & Perspectives," J. Amer. Med. Assoc., 272: 501 [1994]). Indeed, the Centers for Disease Control recently added *E. coli* O157:H7 to their list of reportable diseases ("Public Health Threats," Science 267:1427 [1995]).

C. Nature of Verotoxin-Induced Disease

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Risk factors for HUS progression following infection with *E. coli* O157:H7 include age (very young or elderly), bloody diarrhea, leukocytosis, fever, large amounts of ingested pathogen, previous gastrectomy, and the use of antimicrobial agents (in particular, trimethoprim-sulfamethoxazole)(A. A. Harris *et al.*, "Results of a screening method used in a 12 month stool survey for *Escherichia coli* O157:H7." J. Infect. Dis., 152: 775-777 [1985]; and M. A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*." Clin. Microbiol. Rev., 2: 15-38 [1989]).

As indicated above, *E. coli* O157:H7 is associated with significant morbidity and mortality. The spectrum of illness associated with *E. coli* O157:H7 infection includes asymptomatic infection, mild uncomplicated diarrhea, hemorrhagic colitis, HUS, and TTP". Hemorrhagic colitis (or "ischemic colitis") is a distinct clinical syndrome characterized by

sudden onset of abdominal cramps—likened to the pain associated with labor or appendicitis—followed within 24 hours by watery diarrhea. One to two days later, the diarrhea turns grossly bloody in approximately 90% of patients and has been described as "all blood and no stool" (C. H. Pai et al., "Sporadic cases of hemorrhagic colitis associated with Escherichia coli O157:H7," Ann. Intern. Med., 101: 738-742 [1984]; and R. S. Remis et al., "Sporadic cases of hemorrhagic colitis associated with Escherichia coli O157:H7," Ann. Intern. Med., 101: 738-742 [1984]). Vomiting may occur, but there is little or no fever. The time from ingestion to first loose stool ranges from 3-9 days (with a mean of 4 days) L. W. Riley et al., "Hemorrhagic colitis associated with a rare Escherichia coli serotype." New Eng. J. Med., 308: 681-685 [1983]; and D. Pudden et al., "Hemorrhagic colitis in a nursing home." Ontario Can. Dis. Weekly Rpt., 11: 169-170 [1985]), and the duration of illness ranges generally from 2-9 days (with a mean of 4 days).

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HUS is a life-threatening blood disorder that appears within 3–7 days following onset of diarrhea in 10–15% of patients. Those younger than 10 years and the elderly are at particular risk. Symptoms include renal glomerular damage, hemolytic anemia (rupturing of erythrocytes as they pass through damaged renal glomeruli), thrombocytopenia and acute kidney failure. Approximately 15% of patients with HUS die or suffer chronic renal failure. Indeed, HUS is a leading cause of renal failure in childhood (reviewed by M.A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*," Clin. Microbiol. Rev., 2: 15-38 [1989]). Currently, blood transfusion and dialysis are the only therapies for HUS.

TTP shares similar histopathologic findings with HUS, but usually results in multiorgan microvascular thrombosis. Neurological signs and fever are more prominent in TTP, compared with HUS. Generally occurring in adults, TTP is characterized by microangiopathic hemolytic anemia, profound thrombocytopenia, fluctuating neurologic signs, fever and mild azotemia (H. C. Kwaan, "Clinicopathological features of thrombotic thrombocytopenic purpura," Semin, Hematol., 24: 71-81 [1987]; and S. J. Machin, "Clinical annotation: Thrombotic thrombocytopenic purpura," Br. J. Hematol., 56: 191-197 [1984]). Patients often die from microthrombi in the brain. In one review of 271 cases, a rapidly progressive course was noted, with 75% of patients dying within 90 days (E.L. Amorosi and J.E. Ultmann, "Thrombotic thrombocytopenic purpura: Report of 16 cases and review of the literature," Med., 45:139-159 (1966).

Other diseases associated with E. coli O157:H7 infection include hemorrhagic cystitis and balantitis (W. R. Grandsen et al., "Hemorrhagic cystitis and balantitis associated with

verotoxin-producing Escherichia coli O157:H7," Lancet ii: 150 [1985]), convulsions, sepsis with other organisms and anemia (P. C. Rowe et al., "Hemolytic anemia after childhood Escherichia coli O157:H7 infection: Are females at increased risk?" Epidemiol. Infect., 106: 523-530 [1991]).

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D. Mechanism of Pathogenesis

Verotoxins are strongly linked to *E. coli* O157:H7 pathogenesis. All clinical isolates of *E. coli* O157:H7 have been shown to produce one or both verotoxins (VT1 and VT2) (C. A. Bopp *et al.*, "Unusual Verotoxin-producing *Escherichia coli* associated with hemorrhagic colitis." J. Clin. Microbiol., 25: 1486-1489 [1987]). Both of these toxins are cytotoxic to Vero (African green monkey kidney) and HeLa cells. and cause paralysis and death in mice (A. D. O'Brien *et al.*, "Purification of *Shigella dysenteriae* 1 (Shiga) like toxin from *Escherichia coli* O157:H7 strain associated with hemorrhagic colitis." *Lancet* ii: 573 [1983]). These toxins are sometimes referred to in the literature as Shiga-like toxins 1 and II (SLT-I and SLT-II, respectively), due to their similarities with the toxins produced by *Shigella*. Indeed, much of our understanding of *E. coli* VTs is based on information accumulated on Shiga toxins. Shiga toxin, first described in 1903, has been recognized as one of the most potent bacterial toxins for eukaryotic cells (reviewed by M.A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*," Clin. Microbiol. Rev., 2: 15-38 [1989]). Hereinafter, the VT convention will be used: thus, VT1 and VT2 correspond to SLT-I and SLT-II, respectively.

While the pathogenic mechanism of *E. coli* O157:H7 infection is incompletely understood, it is believed that ingested organisms adhere to and colonize the intestinal mucosa, where toxins are released which cause endothelial cell damage and bloody diarrhea. It is also postulated that hemorrhagic colitis progresses to HUS when verotoxins enter the bloodstream, damaging the endothelial cells of the microvasculature and triggering a cascade of events resulting in thrombus deposition in small vessels. These microthrombi occlude the microcapillaries of the kidneys (particularly in the glomeruli) and other organs, resulting in their failure (J. J. Byrnes and J. L. Moake, "TTP and HUS syndrome: Evolving concepts of pathogenesis and therapy." Clin. Hematol., 15: 413-442 [1986]; and T. G. Cleary, "Cytotoxin-producing *Escherichia coli* and the hemolytic uremic syndrome." Pediatr. Clin. North Am., 35: 485-501 [1988]). Verotoxins entering the bloodstream may also result in direct kidney cytotoxicity.

VT1 is immunologically and structurally indistinguishable from Shiga toxin produced by Shigella dysenteriae (A. D. O'Brien et al., "Purification of Shigella dysenteriae 1 (Shiga) like toxin from Escherichia coli O157:H7 strain associated with hemorrhagic colitis." Lancet ii: 573 [1983]). VT1 and VT2 holotoxins each consist of one A and five B subunits (A. Donohue-Rolfe et al., "Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross reactive monoclonal antibodies." Infect. Immun., 57: 3888-3893 [1989]: and A. Donohue-Rolfe et al., "Simplified high yield purification of Shigella toxin and characterization of subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies." J. Exp. Med., 160: 1767-1781 [1984]). The toxic A subunit is enzymatically active, while the B subunit binds the holotoxin to the receptor on the target eukaryotic cell.

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Crystal structure analysis of Shiga holotoxin and VT1 B subunit pentamers have shown that the holotoxin assembles with the C-terminal end of the A subunit associating with, and inserting within, a pentamer of B chains (P. E. Stein et al., "Crystal structure of the cell-binding B oligomer of verotoxin-1 from E. coli," Nature 355: 748-750 [1992]; and M.E. Fraser et al., "Crystal structure of the holotoxin from Shigella dysenteriae at 2.5 Å resolution," Struct. Biol., 1:59-64 [1994]). This conformation is consistent with the observation that a C-terminally truncated A1 subunit of VT1 is toxic (in a ribosomal inhibition assay), but cannot associate with B subunit pentamers (P. R. Austin et al., "Evidence that the A₂ fragment of Shiga-like toxin type 1 is required for holotoxin integrity," Infect, Immun., 62: 1768 [1994]).

The Verotoxin A Subunit. Examination of the crystal structure of Shiga holotoxin indicates that the N-terminus of its A subunit is both surface-exposed and functionally important. Removal of amino acid interval 3–18 of the A subunit completely abolished toxicity (L. P. Perera et al., "Mapping the minimal contiguous gene segment that encodes functionally active Shiga-like toxin II." Infect. Immun., 59: 829-835 [1991]) while removal of interval 25–44 retained toxicity but abolished its association with B subunit pentamers (J. E. Haddad et al., "Minimum domain of the Shiga toxin A subunit required for enzymatic activity." J. Bacteriol., 175: 4970-4978 [1993]). Deletion of the first 13 residues of the homologous ricin A subunit also abolished toxicity, while deletion of the first 9 residues did not (M. J. May, et al., "Ribosome inactivation by ricin A chain: A sensitive method to assess the activity of wild-type and mutant polypeptides." EMBO J., 8: 301-308 [1989]).

The Verotoxin B Subunit. Studies of Shiga toxin B subunit suggest that neutralizing epitopes may also be present at both the N- and C-terminal regions of VT1 and VT2 B

subunits. Polyclonal antibodies raised against peptides from these regions (residues 5-18. 13-26. 7-26. 54-67 and 57-67) show partial neutralization of Shiga toxin (1. Harari and R. Arnon. "Carboxy-terminal peptides from the B subunit of Shiga toxin induce a local and parenteral protective effect." Mol. Immunol., 27: 613-621 [1990]; and I. Harari *et al.*. "Synthetic peptides of Shiga toxin B subunit induce antibodies which neutralize its biological activity." Infect. Immun., 56: 1618-1624 [1988]). Deletion of the last five amino acids of Shiga toxin B (M. P. Jackson *et al.*. "Functional Analysis of the Shiga toxin and Shiga-like toxin Type II variant binding subunits by using site-directed mutagenesis." J. Bacteriol., 172: 653-658 [1990]), or four amino acids of VT2 B (L. P. Perera *et al.*. "Mapping the minimal contiguous gene segment that encodes functionally active Shiga-like toxin II." Infect. Immun., 59: 829-835 [1991]), eliminate toxin activity, while deletion of the last two amino acids of VT2 B subunit reduced cytotoxicity. In contrast, the addition of an 18 or 21 amino acid extension to the native C-terminus of the VT2 B subunit was presumably conformationally correct, as these proteins assembled cytotoxic holotoxin.

Various approaches to express recombinant verotoxins have included individual or

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coordinate expression of A and B subunits from high-copy number plasmids and expression with fusion partners (J. E. Haddad et al., "Minimum domain of the Shiga toxin A subunit required for enzymatic activity." J. Bacteriol., 175: 4970-4978; J. E. Haddad, and M. P. Jackson. "Identification of the Shiga toxin A-subunit residues required for holotoxin assembly." J. Bacteriol., 175: 7652-7657 [1993]; M. P. Jackson et al., "Mutational analysis of the Shiga toxin and Shiga-like toxin II enzymatic subunits," J. Bacteriol., 172: 3346-3350 [1990]; C. J. Hoyde et al., "Evidence that glutamic acid 167 is an active-site residue of Shigalike toxin I." Proc. Natl. Acad. Sci., 85: 2568-2572 [1988]; R. L. Deresiewicz et al., "The role of tyrosine-114 in the enzymatic activity of the Shiga-like toxin I A-chain." Mol. Gen. Genet., 241: 467-473 [1993]; T. M. Zollman et al., "Purification of Recombinant Shiga-like Toxin Type I A, Fragment from Escherichia coli." Protein Express. Purific.. 5: 291-295 [1994]: K. Ramotar, et al., "Characterization of Shiga-like toxin I B subunit purified from overproducing clones of the SLT-I B cistron." Biochem J., 272: 805-811 [1990]; S. B. Calderwood et al.. "A system for production and rapid purification of large amounts of the Shiga toxin/Shiga-like toxin I B subunit," Infect. Immun., 58: 2977-2982 [1990]; D. W. K. Acheson, et al., "Comparison of Shiga-like toxin I B-subunit expression and localization in Escherichia coli and Vibrio cholerae by using tre or Iron-regulated promoter systems." Infect. Immun. 61: 1098-1104 [1993]; M. P. Jackson et al., "Nucleotide sequence analysis and

comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacgeriophages from *Escherichia coli* 933," FEMS Microbiol. Lett., 44: 109-114 [1987]; J. W. Newland *et al.*. "Cloning of genes for production of *Escherichia coli* Shiga-like toxin type II." Infect. Immun. 55: 2675-2680 [1987]; and F. Gunzer and H. Karch. "Expression of A and B subunits of Shiga-like toxin II as fusions with glutathione *S*-transferase and their potential for use in seroepidemiology.". J. Clin. Microbiol.. 31: 2604-2610 [1993]: and D.W. Acheson *et al.*, "Expression and purification of Shiga-like toxin II B subunits." Inf. Immun.. 63:301-308 [1995]). In one case, bench top fermentation techniques yielded 22 mg/liter of soluble recombinant protein (D. W. K. Acheson, *et al.*. "Comparison of Shiga-like toxin I B-subunit expression and localization in *Escherichia coli* and *Vibrio cholerae* by using *trc* or Iron-regulated promoter systems." Infect. Immun. 61: 1098-1104 [1993]). However, there have been no systematic approaches to identifying the appropriate spectrum of VT antigens.

The receptor for VT1 and VT2 is a globotriaosyl ceramide containing a galactose α-(1-4)- galactose-β-(1-4) glucose ceramide (Gb3) (C. A. Lingwood *et al.*, "Glycolipid binding of natural and recombinant *Escherichia coli* produced verotoxin *in vitro*," J. Biol. Chem., 262: 1779-1785 [1987]; and T. Wadell *et al.*, "Globotriaosyl ceramide is specifically recognized by the *Escherichia coli* verocytotoxin 2." Biochem. Biophys. Res. Commun., 152: 674-679 [1987]). Gb3 is abundant in the cortex of the human kidney and is present in primary human endothelial cell cultures. Hence, the identification of Gb3 as the functional receptor for VT1 and VT2 is consistent with their role in HUS pathogenesis, in which endothelial cells of the renal vasculature are the principal site of damage. Therefore, toxin-mediated pathogenesis may follow a sequence of B subunit binding to Gb3 receptors on kidney cells, toxin internalization, enzymatic reduction of the A subunit to an A1 fragment, binding of the A1 subunit to the 60S ribosomal subunit, inhibition of protein synthesis and cell death (A. D. O'Brien *et al.*, "Shiga and Shiga-like toxins, Microbial Rev., 51: 206-220 [1987]).

The role of verotoxins in the pathogenesis of *E. coli* O157:H7 infections has been further studied in animal models. Infection or toxin challenge of laboratory animals do not produce all the pathologies and symptoms of hemorrhagic colitis. HUS, and TTP which occur in humans. Glomerular damage is noticeably absent. Nonetheless, experiments using animal models implicate verotoxins as the direct cause of hemorrhagic colitis, microvascular damage leading to the failure of kidneys and other organs and CNS neuropathies.

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For example, Barrett, et al. delivered VT2 into the peritoneal cavity of rabbits using mini-osmotic pumps (J. J. Barrett et al., "Continuous peritoneal infusion of shiga-like toxin II (SLTII) as a model for SLT II-induced diseases." J. Infect. Dis., 159: 774-777 [1989]). In three days, most animals receiving the toxin developed diarrhea, with intestinal lesions resembling those seen in humans with hemorrhagic colitis. Although there was some evidence of renal dysfunction, none of the rabbits developed HUS. Beery, et al. showed that VT2, when administered intraperitoneally or intravenously to adult mice, produces lesions of the kidneys and colon (J. T. Beery et al., "Cytotoxic activity of Escherichia coli O157:H7 culture filtrate on the mouse colon and kidney." Curr. Microbiol., 11: 335-342 [1984]). Histologic lesions in the kidney included accumulation of numerous exfoliated collecting tubules and marked intracellular vacuolation of proximal convoluted tubular cells.

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Sjögren et. al. studied the pathogenesis of an entero-adherent strain of E. coli (RDEC-1) lysogenized with a VT1-containing bacteriophage (VT1-producing RDEC-1) (R. Sjögren et al., "Role of Shiga-like toxin I in bacterial enteritis: comparison between isogenic Escherichia coli strains induced in rabbits." Gastroenterol., 106: 306-317 [1994]). In this study, rabbits were challenged with RDEC-1 or VT1-producing RDEC-1 and studied for onset of disease. The VT1-producing variant induced a severe, non-invasive, entero-adherent infection in rabbits which was characterized by serious histological lesions with vascular changes, edema and severe epithelial inflammation. Importantly, vascular changes consistent with endothelial damage were seen in infected animals that was similar to intestinal microvascular changes in humans with E. coli O157:H7 infection. Based on these observations, they concluded that VT1 is an important virulence factor in enterohemorrhagic E. coli O157:H7 infection.

Fuji et. al. described a model in which mice were treated for three days with streptomycin followed by a simultaneous challenge of E. coli O157:H7 orally, and mitomycin intraperitoneally (J. Fuji et al., "Direct evidence of neuron impairment by oral infection with Verotoxin-producing Escherichia coli O157:H7 in mitomycin-treated mice." Infect. Immun. 62: 3447-34453 [1994]). All of the animals died within four days. Immunoelectron-microscopy strongly suggested that death was due to the toxic effects of VT2v (a structural variant of VT2), on both the endothelial cells and neurons in the central nervous system which resulted in fatal acute encephalopathy.

Wadolkowski et al. studied colonization of E. coli O157:H7 in mice. Mice were treated with streptomycin and fed 10¹⁰ E. coli O157:H7 (E. A. Wadolkowski et al. "Mouse

model for colonization and disease caused by enterohemorrhagic Escherichia coli O157:H7." Infect. Immun.. 58: 2438-2445 [1990]: and E. A. Wadolkowski et al., "Acute renal tubular necrosis and death of mice orally infected with Escherichia coli strains that produce Shigalike toxin Type II." Infect. Immun.. 58: 3959-3965 [1990]). All of the mice died due to severe, disseminated, acute necrosis of proximal convoluted tubules. In mouse models, glomerular damage was not observed, but toxic acute renal tubular necrosis was observed which is characteristic of some HUS patients. The failure of mice to show glomerular damage is thought to be due to the absence of a functional globotriaosyl ceramide receptor specific for verotoxins in the glomeruli of the kidneys. Administration of VT2 subunit-specific monoclonal antibodies prior to infection prevented all pathology and death.

E. Current Therapeutic Approaches

E. coli O157:H7 disease is not adequately controlled by current therapy. Patient treatment is tailored to manage fluid and electrolyte disturbances, anemia, renal failure and hypertension. Although E. coli O157:H7 is susceptible to common antibiotics, the role of antibiotics in the treatment of infection has questionable merit. In both retrospective and prospective studies, prophylaxis or treatment with antibiotics such as trimethoprimsulfamethoxazole, there was either no benefit or an increased risk of developing HUS (T. N. Bokete et al., "Shiga-like toxin producing Escherichia coli in Seattle children: a prospective study," Gastroenterol., 105: 1724-1731 [1993]; A. T. Pavia et al., "Hemolytic uremicsyndrome during an outbreak of Escherichia coli O157:H7 infections in institutions for mentally retarded persons: clinical and epidemiologic observations." J. Pedatr., 116: 544-551 [1990]; F. Proulx et al., "Randomized, controlled trial of antibiotic therapy for Escherichia coli O157:H7 enteritis." J. Pediatr., 121: 299-303 [1992]; and A. L. Carter et al., "A severe outbreak of Escherichia coli O157:H7-associated hemorrhagic colitis in a nursing home." New Eng. J. Med., 317: 1496-1500 [1987]).

The mechanisms by which antibiotics increase the risk of infection or related complications might involve enhancement of toxin production, release of toxins from killed organisms, or alteration of normal competing intestinal flora allowing for pathogen overgrowth (M. A. Karmali, "Infection by Verocytotoxin-producing Escherichia coli," Clin. Microbiol. Rev., 2: 15-38 [1989]). A further concern in the use of antibiotics is the potential acquisition of antimicrobial resistance by E. coli O157:H7 (C. R. Dorn, "Review of foodborne outbreak of Escherichia coli O157:H7 infection in the western United States," JAVMA 203: 1583-1587 [1993]).

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In addition, by the time symptoms are serious enough to attract medical attention, it is likely that verotoxins are already entering the systemic circulation or will do so shortly thereafter. Although antimicrobials may help to prevent pathology resulting from the action of toxin on the bowel lumen. However, by the time symptoms of HUS have developed, the patient has ceased shedding organisms. Thus, antimicrobial treatment during HUS disease is of less value, and often contraindicated, due to the increased risk of complications associated with administration of antimicrobials to patients susceptible to development of HUS. Importantly, there is currently no antitoxin commercially available for use in treating affected patients. What is needed is a means to block the progression of disease, without the complications associated with antimicrobial treatment.

DESCRIPTION OF THE DRAWINGS

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Figure 1 is an SDS-PAGE of rVT1 and rVT2.

Figure 2 shows HPLC results for rVT1 and rVT2.

Figure 3 shows rVT1 and rVT2 toxicity in Vero cell culture.

Figure 4 shows EIA reactivity of rVT1 and rVT2 antibodies to rVT1.

Figure 5 shows EIA reactivity of rVT1 and rVT2 Antibodies to rVT2.

Figure 6 shows Western Blot reactivity of rVT1 and rVT2 antibodies to rVT's:

Panel 6A contains preimmune lgY:

Panel 6B contains rVT1 IgY; and

Panel 6C contains rVT2 IgY.

Figure 7 shows neutralization of rVT1 cytotoxicity in Vero cells.

Figure 8 shows neutralization of rVT2 cytotoxicity in Vero cells.

Figure 9 shows renal sections from E. coli O157:H7-infected mice treated with lgY

Panel 9A shows a representative kidney section from a mouse treated with preimmune lgY;

Panel 9B shows a representative kidney sections from a mouse treated with rVT1; and

Panel 9C shows a representative kidney section from a mouse treated with rVT2 IgY.

Figure 10 shows the fusion constructs of VT components and affinity tags.

DEFINITIONS

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To facilitate understanding of the invention, a number of terms are defined below.

As used herein, the term "neutralizing" is used in reference to antitoxins, particularly antitoxins comprising antibodies, which have the ability to prevent the pathological actions of the toxin against which the antitoxin is directed.

As used herein, the term "overproducing" is used in reference to the production of toxin polypeptides in a host cell, and indicates that the host cell is producing more of the toxin by virtue of the introduction of nucleic acid sequences encoding the toxin polypeptide than would be expressed by the host cell absent the introduction of these nucleic acid sequences. To allow ease of purification of toxin polypeptides produced in a host cell it is preferred that the host cell express or overproduce the toxin polypeptide at a level greater than I mg/liter of host cell culture.

As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (i.e., an E. coli verotoxin and/or fragments thereof) joined to an exogenous protein fragment (the fusion partner which consists of a non-toxin protein). The fusion partner may enhance solubility of the E. coli protein as expressed in a host cell, may provide an "affinity tag" to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both. If desired, the fusion protein may be removed from the protein of interest (i.e., toxin protein or fragments thereof) prior to immunization by a variety of enzymatic or chemical means known to the art.

As used herein, the term "affinity tag" refers to such structures as a "poly-histidine tract" or "poly-histidine tag," or any other structure or compound which facilitates the purification of a recombinant fusion protein from a host cell, host cell culture supernatant, or both. As used herein, the term "flag tag" refers to short polypeptide marker sequence useful for recombinant protein identification and purification.

As used herein, the terms "poly-histidine tract" and "poly-histidine tag," when used in reference to a fusion protein refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus of a protein of interest. A poly-histidine tract of six to ten residues is preferred. The poly-histidine tract is also defined functionally as being a number of consecutive histidine residues added to the protein of interest which allows the affinity purification of the resulting fusion protein on a nickel-chelate column.

As used herein, the term "chimeric protein" refers to two or more coding sequences obtained from different genes, that have been cloned together and that, after translation, act as

a single polypeptide sequence. Chimeric proteins are also referred to as "hybrid proteins."

As used herein, the term "chimeric protein" refers to coding sequences that are obtained from different species of organisms, as well as coding sequences that are obtained from the same species of organisms.

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As used herein, the term "protein of interest" refers to the protein whose expression is desired within the fusion protein. In a fusion protein, the protein of interest will be joined or fused with another protein or protein domain, the fusion partner, to allow for enhanced stability of the protein of interest and/or ease of purification of the fusion protein.

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As used herein, the term "maltose binding protein" and "MBP" refers to the maltose binding protein of *E. coli*. A portion of the maltose binding protein may be added to a protein of interest to generate a fusion protein; a portion of the maltose binding protein may merely enhance the solubility of the resulting fusion protein when expressed in a bacterial host. On the other hand, a portion of the maltose binding protein may allow affinity purification of the fusion protein on an amylose resin.

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As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, antitoxins are purified by removal of contaminating non-immunoglobulin proteins: they are also purified by the removal of substantially all immunoglobulin that does not bind toxin. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind toxin results in an increase in the percent of toxin-reactive immunoglobulins in the sample. In another example, recombinant toxin polypeptides are expressed in bacterial host cells and the toxin polypeptides are purified by the removal of host cell proteins: the percent of recombinant toxin polypeptides is thereby increased in the sample.

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The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

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The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

The term "native protein" as used herein refers to a protein which is isolated from a

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As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

natural source as opposed to the production of a protein by recombinant means.

As used herein "soluble" when in reference to a protein produced by recombinant DNA technology in a host cell, is a protein which exists in solution in the cytoplasm of the host cell; if the protein contains a signal sequence, the soluble protein is exported to the periplasmic space in bacterial hosts and is secreted into the culture medium of eukaryotic cells capable of secretion or by bacterial hosts possessing the appropriate genes. In contrast, an insoluble protein is one which exists in denatured form inside cytoplasmic granules (called an inclusion bodies) in the host cell. High level expression (i.e., greater than 1 mg recombinant protein/liter of bacterial culture) of recombinant proteins often results in the expressed protein being found in inclusion bodies in the bacterial host cells. A soluble protein is a protein which is not found in an inclusion body inside the host cell or is found both in the cytoplasm and in inclusion bodies and in this case the protein may be present at high or low levels in the cytoplasm.

A distinction is drawn between a soluble protein (i.e., a protein which when expressed in a host cell is produced in a soluble form) and a "solubilized" protein. An insoluble recombinant protein found inside an inclusion body may be solubilized (i.e., rendered into a soluble form) by treating purified inclusion bodies with denaturants such as guanidine hydrochloride, urea or sodium dodecyl sulfate (SDS). These denaturants must then be removed from the solubilized protein preparation to allow the recovered protein to renature (refold). Not all proteins will refold into an active conformation after solubilization in a denaturant and removal of the denaturant. Many proteins precipitate upon removal of the denaturant. SDS may be used to solubilize inclusion bodies and will maintain the proteins in solution at low concentration. However, dialysis will not always remove all of the SDS (SDS can form micelles which do not dialyze out): therefore, SDS-solubilized inclusion body protein is soluble but not refolded.

As used herein, the term "reporter reagent" or "reporter molecule" is used in reference to compounds which are capable of detecting the presence of antibody bound to antigen. For example, a reporter reagent may be a colorimetric substance which is attached to an enzymatic substrate. Upon binding of antibody and antigen, the enzyme acts on its substrate and causes the production of a color. Other reporter reagents include, but are not limited to fluorogenic and radioactive compounds or molecules.

As used herein the term "signal" is used in reference to the production of a sign that a reaction has occurred, for example, binding of antibody to antigen. It is contemplated that signals in the form of radioactivity, fluorogenic reactions, and enzymatic reactions will be

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used with the present invention. The signal may be assessed quantitatively as well as qualitatively.

As used herein, the term "therapeutic amount" refers to that amount of antitoxin required to neutralize the pathologic effects of *E. coli* toxin in a subject.

As used herein, the term "acute intoxication" is used in reference to cases of *E. coli* infection in which the patient is currently suffering from the effects of toxin (e.g., E. coli verotoxins or enterotoxins). Signs and symptoms of intoxication with the toxin may be immediately apparent. Or, the determination of intoxication may require additional testing, such as detection of toxin present in the patient's fecal material.

As used herein, the term "at risk" is used in references to individuals who have been exposed to *E. coli* and may suffer the symptoms associated with infection or disease with these organisms, especially due to the effects of verotoxins.

SUMMARY OF THE INVENTION

The present invention relates to antitoxin therapy for humans and other animals. Antitoxins which neutralize the pathologic effects of *E. coli* toxins are generated by immunization of avian hosts with recombinant toxin fragments. In one embodiment, the present invention contemplates a method of treatment administering at least one antitoxin directed against at least a portion of an *Escherichia coli* verotoxin in an aqueous solution in therapeutic amount that is administrable to an intoxicated subject. It is contemplated that the intoxicated subject will be either an adult or a child.

In a preferred embodiment, the *E. coli* verotoxin is recombinant. In one embodiment, the antitoxin is an avian antitoxin. In an alternative embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence. In one embodiment of the *E. coli* fusion protein, the fusion protein comprises a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT2 sequence.

Various routes of administration, are contemplated for providing the *E. coli* antitoxin(s) to an affected individual, including but not limited to, parenteral as well as oral routes of administration. In a particularly preferred embodiment, the route of administration is parenteral.

The present invention also includes the embodiment of a method of prophylactic treatment in which an antitoxin directed against at least one *E. coli* verotoxin in an aqueous

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solution in therapeutic amount that is parenterally administrable, and is administered to at least one subject at risk of diarrheal disease. It one embodiment, the antitoxin is parenterally administered.

In one embodiment, the subject is at risk of developing extra-intestinal complications of *E. coli* infections, including but not limited to, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, etc.

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The present invention also includes the embodiment of a composition which comprises neutralizing antitoxin directed against at least one *E. coli* verotoxin in an aqueous solution in therapeutic amounts. In one particularly preferred embodiment, the *E. coli* verotoxin is a recombinant toxin. In an alternative embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *E. coli* verotoxin VT1 sequence. In another embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *E. coli* verotoxin VT2 sequence. In yet another embodiment, the composition of the antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin. In one embodiment, the portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT1. In an alternative embodiment, the portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT2. Indeed, the invention contemplates an antitoxin that is directed against a portion of at least one *Escherichia coli* verotoxin. In one embodiment, the antitoxin is an avian antitoxin.

The present invention also comprises a method of treatment of enteric bacterial infections comprising administering an avian antitoxin directed against at least one verotoxin produced by *E. coli* in an aqueous solution in therapeutic amount, to at least one infected subject. In one preferred embodiment, the avian antitoxin is administered parenterally.

In another embodiment, the *E. coli* is selected from the group consisting of *Escherichia coli* serotypes O157:H7. O1:NM: O2:H5: O2:H7: O4:NM: O4:H10: O5:NM: O5:H16: O6:H1: O18:NM: O18:H7: O25:NM: O26:NM: O26:H11: O26:H32: O38:H21: O39:H4: O45:H2: O50:H7: O55:H7: O55:H10: O82:H8: O84:H2: O91:NM: O91:H21: O103:H2: O111:NM: O111:H8: O111:H30: O111:H34: O113:H7: O113:H21: O114:H48: O115:H10: O117:H4: O118:H12: O118:H30: O121:NM: O121:H19: O125:NM: O125:H8: O126:NM: O126:H8: O128:NM: O128:H2: O128:H2: O128:H2: O128:H25: O145:NM: O125:H25: O146:H21: O153:H25: O157:NM: O163:H19: O165:NM: O165:19: and O165:H25. In one embodiment, the antitoxin comprises antitoxin directed against at least one

Escherichia coli verotoxin. In another embodiment, the antitoxin is cross-reacts with at least one Escherichia coli verotoxin. In yet another embodiment, the antitoxin is reactive against toxins produced by members of the genus Shigella, including S. dysenteriae.

The present invention also contemplates uses for the toxin fragments in vaccines and diagnostic assays. The fragments may be used separately as purified, soluble antigens or, alternatively, in mixtures or "cocktails." The present invention thus comprises a method for detecting Escherichia coli verotoxin in a sample in which a sample an antitoxin raised against Escherichia coli verotoxin, and a reporter reagent capable of binding the antitoxin are provided. The antitoxin is added to the sample, so that the antitoxin binds to the E. coli verotoxin in the sample. In one embodiment, the antitoxin is an avian antitoxin. In an alternative embodiment, the method further comprises the steps of washing unbound antitoxin from the sample, adding at least one reporter reagent to the sample, so that said reporter reagent binds to any antitoxin that is bound, washing the unbound reporter reagent from the sample and detecting the reporter reagent bound to the antitoxin bound to the Escherichia coli verotoxin, so that the verotoxin is detected. In one embodiment, the detecting is accomplished through any means, such as enzyme immunoassay, radioimmunoassay, fluorescence immunoassay, flocculation, particle agglutination, and in viiu chromogenic assay, In one preferred embodiment, the sample is a biological sample. In an alternative preferred embodiment, the sample is an environmental sample.

DESCRIPTION OF THE INVENTION

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The present invention contemplates treating humans and other animals intoxicated with at least one bacterial toxin. It is contemplated that administration of antitoxin will be used to treat patients effected by or at risk of symptoms due to the action of bacterial toxins. It is also contemplated that the antitoxin will be used in a diagnostic assay to detect the presence of toxins in samples. The organisms, toxins and individual steps of the present invention are described separately below.

I. Antibodies Directed Against E. coli and Associated Toxins

A preferred embodiment of the method of the present invention is directed toward obtaining antibodies against various *E. coli* serotypes, their toxins, enzymes or other metabolic by-products, cell wall components, or synthetic or recombinant versions of any of these compounds. It is contemplated that these antibodies will be produced by immunization

of humans or other animals. It is not intended that the present invention be limited to any particular toxin or any species of organism. In one embodiment, toxins from all *E. coli* serotypes are contemplated as immunogens. Examples of these toxins include the verotoxins VT1 and VT2.

It is not intended that antibodies produced against one toxin will only be used against that toxin. It is contemplated that antibodies directed against one toxin may be used as an effective therapeutic against one or more toxin(s) produced by other *E. coli* serotypes, or other toxin producing organisms (e.g., Shigella, Bacillus cereus, Staphylococcus aureus, Streptococcus mutans, Acinetobacter calcoaceticus, Pseudomonas aeruginosa, other Pseudomonas species, Vibrio species, Clostridium species, etc.). It is further contemplated that antibodies directed against the portion of the toxin which binds to mammalian membranes can also be used against other organisms. It is contemplated that these membrane binding domains are produced synthetically and used as immunogens.

II. Obtaining Antibodies In Non-Mammals

A preferred embodiment of the method of the present invention for obtaining antibodies involves immunization. However, it is also contemplated that antibodies may be obtained from non-mammals without immunization. In the case where no immunization is contemplated, the present invention may use non-mammals with preexisting antibodies to toxins as well as non-mammals that have antibodies to whole organisms by virtue of reactions with the administered antigen. An example of the latter involves immunization with synthetic peptides or recombinant proteins sharing epitopes with whole organism components.

In a preferred embodiment, the method of the present invention contemplates immunizing non-mammals with bacterial toxin(s). It is not intended that the present invention be limited to any particular toxin. In one embodiment, toxins from all *E. coli* serotypes are contemplated as immunogens.

A particularly preferred embodiment involves the use of bacterial toxin protein or fragments of toxin proteins produced by molecular biological means (i.e., recombinant toxin proteins). In a preferred embodiment, the immunogen comprises recombinant VT1 and/or VT2.

When immunization is used, the preferred non-mammal is from the class Aves. All birds are contemplated (e.g., duck, ostrich, emu, turkey, etc.). A preferred bird is a chicken. Importantly, chicken antibody does not fix mammalian complement (See H.N. Benson et al.,

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J. Immunol. 87:616 [1961]). Thus, chicken antibody will normally not cause a complement-dependent reaction (A.A. Benedict and K. Yamaga, "Immunoglobulins and Antibody Production in Avian Species," in Comparative Immunology (J.J. Marchaloni, ed.), pp. 335-375. Blackwell, Oxford [1966]). Thus, the preferred antitoxins of the present invention will not exhibit complement-related side effects observed with antitoxins presently known.

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When birds are used, it is contemplated that the antibody will be obtained from either the bird serum or the egg. A preferred embodiment involves collection of the antibody from the egg. Laying hens transport immunoglobulin to the egg yolk ("IgY") in concentrations equal to or exceeding that found in serum (See R. Patterson et al., J. Immunol, 89:272 (1962); and S.B. Carroll and B.D. Stollar, J. Biol. Chem. 258:24 [1983]). In addition, the large volume of egg yolk produced vastly exceeds the volume of serum that can be safely obtained from the bird over any given time period. Finally, the antibody from eggs is more pure and more homogeneous; there is far less non-immunoglobulin protein (as compared to serum) and only one class of immunoglobulin is transported to the yolk.

When considering immunization with toxins, one may consider modification of the toxins to reduce the toxicity. In this regard, it is not intended that the present invention be limited by immunization with modified toxin. Unmodified ("native") toxin is also contemplated as an immunogen.

It is also not intended that the present invention be limited by the type of modification -- if modification is used. The present invention contemplates all types of toxin modification, including chemical and heat treatment of the toxin. In one embodiment, glutaraldehyde treatment of the toxin is contemplated. In an alternative embodiment, formaldehyde treatment of the toxin is contemplated.

It is not intended that the present invention be limited to a particular mode of immunization: the present invention contemplates all modes of immunization, including subcutaneous, intramuscular, intraperitoneal, and intravenous or intravascular injection, as well as *per os* administration of immunogen.

The present invention further contemplates immunization with or without adjuvant. As used herein, the term "adjuvant" is defined as a substance known to increase the immune response to other antigens when administered with other antigens. If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant -- or that the same adjuvant, once used, be used all the time. While the present invention contemplates all types of adjuvant, whether used separately or in combinations, the preferred use of

adjuvant is the use of Complete Freund's Adjuvant followed sometime later with Incomplete Freund's Adjuvant. The invention also contemplates the use of fowl adjuvant commercially available from RIBI, as well as Quil A adjuvant commercially available from Accurate Chemical and Scientific Corporation, and Gerbu adjuvant also commercially available (GmDP: C.C. Biotech Corp.).

When immunization is used, the present invention contemplates a wide variety of immunization schedules. In one embodiment, a chicken is administered toxin(s) on day zero and subsequently receives toxin(s) in intervals thereafter. It is not intended that the present invention be limited by the particular intervals or doses. Similarly, it is not intended that the present invention be limited to any particular schedule for collecting antibody. The preferred collection time is sometime after day 35.

Where birds are used and collection of antibody is performed by collecting eggs, the eggs may be stored prior to processing for antibody. It is preferred that eggs be stored at 4°C for less than one year.

It is contemplated that chicken antibody produced in this manner can be bufferextracted and used analytically. While unpurified, this preparation can serve as a reference for activity of the antibody prior to further manipulations (e.g., immunoaffinity purification).

III. Increasing The Effectiveness Of Antibodies

When purification is used, the present invention contemplates purifying to increase the effectiveness of both non-mammalian antitoxins and mammalian antitoxins. Specifically, the present invention contemplates increasing the percent of toxin-reactive immunoglobulin. The preferred purification approach for avian antibody is polyethylene glycol (PEG) separation.

The present invention contemplates that avian antibody be initially purified using simple, inexpensive procedures. In one embodiment, chicken antibody from eggs is purified by extraction and precipitation with PEG. PEG purification exploits the differential solubility of lipids (which are abundant in egg yolks) and yolk proteins in high concentrations of PEG 8000 (Polson et al., Immunol, Comm. 9:495 [1980]). The technique is rapid, simple, and relatively inexpensive and yields an immunoglobulin fraction that is significantly more pure, in terms of contaminating non-immunoglobulin proteins than the comparable ammonium sulfate fractions of mammalian sera and horse antibodies. The majority of the PEG is removed from the precipitated chicken immunoglobulin by treatment with ethanol. Indeed,

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PEG-purified antibody is sufficiently pure that the present invention contemplates the use of PEG-purified antitoxins in the passive immunization of intoxicated humans and animals.

IV. Treatment

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The present invention contemplates antitoxin therapy for humans and other animals intoxicated by bacterial toxins. A preferred method of treatment is by parenteral administration of antitoxin.

A. Dosage Of Antitoxin

It was noted by way of background that a balance must be struck when administering currently available antitoxin which is usually produced in large animals such as horses: sufficient antitoxin must be administered to neutralize the toxin, but not so much antitoxin as to increase the risk of untoward side effects. These side effects are caused by: i) patient sensitivity to foreign (e.g. horse) proteins; ii) anaphylactic or immunogenic properties of non-immunoglobulin proteins; iii) the complement fixing properties of mammalian antibodies: and/or iv) the overall burden of foreign protein administered. It is extremely difficult to strike this balance when, as noted above, the degree of intoxication (and hence the level of antitoxin therapy needed) can only be approximated.

The present invention contemplates significantly reducing side effects so that this balance is more easily achieved. Treatment according to the present invention contemplates reducing side effects by using PEG-purified antitoxin from birds.

In one embodiment, the treatment of the present invention contemplates the use of PEG-purified antitoxin from birds. The use of yolk-derived, PEG-purified antibody as antitoxin allows for the administration of: 1) non (mammalian)-complement-fixing, avian antibody; 2) a less heterogeneous mixture of non-immunoglobulin proteins; and 3) less total protein to deliver the equivalent weight of active antibody present in currently available antitoxins. The non-mammalian source of the antitoxin makes it useful for treating patients who are sensitive to horse or other mammalian sera.

As is true in cases of botulism, the degree of an individual's exposure to *E. coli* toxin and the prognosis are often difficult to assess, and depend upon a number of factors (*e.g.*, the quantity of contaminated food ingested, the toxigenicity and serotype of *E. coli* strain ingested, etc.). Thus, the clinical presentation of a patient is usually a more important consideration than a quantitative diagnostic test, for determination of dosage in antitoxin

administration. Indeed, for many toxin-associated diseases (e.g., botulism, tetanus, diphtheria, etc.), there is no rapid, quantitative test to detect the presence of the toxin or organism. Rather, these toxin-associated diseases are medical emergencies which mandate immediate treatment. Confirmation of the etiologic agent must not delay the institution of therapy, as the condition of an affected patient may rapidly deteriorate. In addition to the initial treatment with antitoxin, subsequent doses may be indicated, as the patient's disease progresses. The dosage and timing of these subsequent doses is dependent upon the signs and symptoms of disease in each individual patient.

It is contemplated that the administration of antitoxin to an affected individual would involve an initial injection of an approximately 10 ml dose of immune globulin (with less than approximately 1 gram of total protein). In one preferred embodiment, it is contemplated that at least 50% of the initial injection comprises immune globulin. It is also contemplated that more purified immune globulin be used for treatment, wherein approximately 90% of the initial injection comprises immune globulin. When more purified immune globulin is used, it is contemplated that the total protein will be less than approximately 100 milligrams. It is also contemplated that additional doses be given, depending upon the signs and symptoms associated with *E. coli* verotoxin disease progression.

B. Delivery Of Antitoxin

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Although it is not intended to limit the route of delivery, the present invention contemplates a method for antitoxin treatment of bacterial intoxication in which delivery of antitoxin is parenteral or oral.

In one embodiment, antitoxin is parenterally administrated to a subject in an aqueous solution. It is not intended that the parenteral administration be limited to a particular route. Indeed, it is contemplated that all routes of parenteral administration will be used. In one embodiment, parenteral administration is accomplished via intramuscular injection. In an alternative embodiment, parenteral administration is accomplished via intravenous injection.

In another embodiment, antitoxin is delivered in a solid form (e.g., tablets). In an alternative embodiment antitoxin is delivered in an aqueous solution. When an aqueous solution is used, the solution has sufficient ionic strength to solubilize antibody protein, yet is made palatable for oral administration. The delivery solution may also be buffered (e.g., carbonate buffer, pH 9.5) which can neutralize stomach acids and stabilize the antibodies when the antibodies are administered orally. In one embodiment the delivery solution is an

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aqueous solution. In another embodiment the delivery solution is a nutritional formula. Preferably, the delivery solution is infant or a dietary supplement formula (e.g., Similac&, Ensure&, and Enfamil®). Yet another embodiment contemplates the delivery of lyophilized antibody encapsulated or microencapsulated inside acid-resistant compounds.

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Methods of applying enteric coatings to pharmaceutical compounds are well known to the art (companies specializing in the coating of pharmaceutical compounds are available: for example. The Coating Place [Verona, WI] and AAI [Wilmington, NC]). Enteric coatings which are resistant to gastric fluid and whose release (i.e., dissolution of the coating to release the pharmaceutical compound) is pH dependent are commercially available (for example, the polymethacrylates Eudragit® L and Eudragit® S [Röhm Tech Inc., Malden, MA]). Eudragit® S is soluble in intestinal fluid from pH 7.0; this coating can be used to microencapsulate lyophilized antitoxin antibodies and the particles are suspended in a solution having a pH above or below pH 7.0 for oral administration. The microparticles will remain intact and undissolved until they reached the intestines where the intestinal pH would cause them to dissolve thereby releasing the antitoxin.

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The invention contemplates a method of treatment which can be administered for treatment of acute intoxication. In one embodiment, antitoxin is administered orally in either a delivery solution or in tablet form, in therapeutic dosage, to a subject intoxicated by the bacterial toxin which served as immunogen for the antitoxin. In another embodiment of treatment of acute intoxication, a therapeutic dosage of the antitoxin in a delivery solution, is parenterally administered.

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The invention also contemplates a method of treatment which can be administered prophylactically. In one embodiment, antitoxin is administered orally, in a delivery solution, in therapeutic dosage, to a subject, to prevent intoxication of the subject by the bacterial toxin which served as immunogen for the production of antitoxin. In another embodiment, antitoxin is administered orally in solid form such as tablets or as microencapsulated particles. Microencapsulation of lyophilized antibody using compounds such as Eudragit® (Rohm GmbH) or polyethylene glycol, which dissolve at a wide range of pH units, allows the oral administration of solid antitoxin in a liquid form (i.e., a suspension) to recipients unable to tolerate administration of tablets (e.g., children or patients on feeding tubes). In one preferred embodiment the subject is a child. In another embodiment, antibody raised against whole bacterial organism is administered orally to a subject, in a delivery solution, in therapeutic

dosage. In yet another preferred embodiment of prophylactic treatment, a therapeutic dosage of the antitoxin in a delivery solution, is parenterally administered.

V. Multivalent Vaccines Against E. coli Strains

The invention contemplates the generation of multivalent vaccines for the protection of an organism (particularly humans) against several *E. coli* strains. Of particular interest is a vaccine which stimulates the production of a humoral immune response to *E. coli* O157:H7. O26:H11. O113:H21. O91:H21. and O111:NM, in humans. The antigens comprising the vaccine preparation may be native or recombinantly produced toxin proteins from the *E. coli* serotypes listed above. When native toxin proteins are used as immunogens they are generally modified to reduce the toxicity. It is contemplated that glutaraldehyde-modified toxin proteins will be used. In an alternative embodiment, is formaldehyde-modified toxin proteins will be used.

The invention contemplates that recombinant *E. voli* verotoxin proteins be used in conjunction with either native toxins or toxoids from other organisms as antigens in a multivalent vaccine preparation. It is also contemplated that recombinant *E. voli* toxin proteins be used in the multivalent vaccine preparation.

VI. Detection Of Toxin

The invention contemplates detecting bacterial toxin in a sample. The term "sample" in the present specification and claims is used in its broadest sense. On the one hand it is meant to include a specimen or culture (e.g., microbiological cultures). On the other hand, it is meant to include both biological and environmental samples.

Biological samples may be animal, including human, fluid, solid (e.g., stool) or tissue, as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may be obtained from all of the various families of common domestic animals, including but not limited, to bovines (e.g., cattle), ovines (e.g., sheep), caprines (e.g., goats), porcines (e.g., swine), equines (e.g., horses), canines (e.g., dogs), lagamorphs (e.g., rabbits), and felines (e.g., cats), etc. It is also intended that samples may be obtained from feral or wild animals, including, but not limited to, such animals as ungulates (e.g., deer), bear, fish, lagamorphs, rodents, etc.

Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing

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instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

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The invention contemplates detecting bacterial toxin by a competitive immunoassay method that utilizes recombinant toxin VT1 and toxin VT2 proteins, antibodies raised against recombinant bacterial toxin proteins. A fixed amount of the recombinant toxin proteins are immobilized to a solid support (e.g., a microtiter plate) followed by the addition of a biological sample suspected of containing a bacterial toxin. The biological sample is first mixed with affinity-purified or PEG fractionated antibodies directed against the recombinant toxin protein. A reporter reagent is then added which is capable of detecting the presence of antibody bound to the immobilized toxin protein. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. If toxin is present in the sample, this toxin will compete with the immobilized recombinant toxin protein for binding to the anti-recombinant antibody thereby reducing the signal obtained following the addition of the reporter reagent. A control is employed where the antibody is not mixed with the sample. This gives the highest (or reference) signal.

The invention also contemplates detecting bacterial toxin by a "sandwich" immunoassay method that utilizes antibodies directed against recombinant bacterial toxin proteins. Affinity-purified antibodies directed against recombinant bacterial toxin proteins are immobilized to a solid support (e.g., microtiter plates). Biological samples suspected of containing bacterial toxins are then added followed by a washing step to remove substantially all unbound antitoxin. The biological sample is next exposed to the reporter substance, which binds to antitoxin and is then washed free of substantially all unbound reporter substance. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. Identification of the reporter substance in the biological tissue indicates the presence of the bacterial toxin.

It is also contemplated that bacterial toxin be detected by pouring liquids (e.g., soups and other fluid foods and feeds including nutritional supplements for humans and other animals) over immobilized antibody which is directed against the bacterial toxin. It is contemplated that the immobilized antibody will be present in or on such supports as cartridges, columns, beads, or any other solid support medium. In one embodiment, following

the exposure of the liquid to the immobilized antibody, unbound toxin is substantially removed by washing. The liquid is then exposed to a reporter substance which detects the presence of bound toxin. In a preferred embodiment the reporter substance is an enzyme, fluorescent dye, or radioactive compound attached to an antibody which is directed against the toxin (i.e., in a "sandwich" immunoassay). It is also contemplated that the detection system will be developed as necessary (e.g., the addition of enzyme substrate in enzyme systems: observation using fluorescent light for fluorescent dye systems: and quantitation of radioactivity for radioactive systems).

EXPERIMENTAL

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The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the disclosure which follows, the following abbreviations apply: °C (degrees Centigrade): rpm (revolutions per minute): BSA (bovine serum albumin): ELISA (enzymelinked immunosorbent assay): IgG (immunoglobulin G); IgY (immunoglobulin Y); IP (intraperitoneal): SC (subcutaneous): H₂O (water): HCl (hydrochloric acid): LD₁₀₀ (lethal dose for 100% of experimental animals); aa (amino acid); HPLC (high performance liquid chromatography): Kda (kilodaltons): gm (grams): µg (micrograms): mg (milligrams): ng (nanograms): µl (microliters): ml (milliliters): mm (millimeters): nm (nanometers): µm (micrometer): M (molar): mM (millimolar): MW (molecular weight): sec (seconds): min(s) (minute/minutes): hr(s) (hour/hours): MgCl₂ (magnesium chloride): NaCl (sodium chloride): Na₂CO₃ (sodium carbonate): OD₂₈₀ (optical density at 280 nm): OD₆₀₈₁ (optical density at 600 nm): PAGE (polyacrylamide gel electrophoresis): PBS [phosphate buffered saline (150 mM NaCl. 10 mM sodium phosphate buffer, pH 7.2)]; PEG (polyethylene glycol); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); w/v (weight to volume); v/v (volume to volume): Amicon (Amicon, Inc., Beverly, MA): Amresco (Amresco, Inc., Solon, OH): ATCC (American Type Culture Collection, Rockville, MD): BBL (Baltimore Biologics Laboratory. (a division of Becton Dickinson). Cockeysville. MD): Becton Dickinson (Becton Dickinson Labware, Lincoln Park, NJ); BioRad (BioRad, Richmond, CA); Biotech (C-C Biotech Corp., Poway, CA): Charles River (Charles River Laboratories, Wilmington, MA): Falcon (e.g. Baxter Healthcare Corp., McGaw Park, IL and Becton Dickinson): Fisher Biotech (Fisher Biotech, Springfield, NJ); GIBCO (Grand Island Biologic Company/BRL, Grand Island, NY); Mallinckrodi (a division of Baxter Healthcare Corp., McGaw Park, IL);

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Millipore (Millipore Corp., Marlborough, MA): New England Biolabs (New England Biolabs, Inc., Beverly, MA): Novagen (Novagen, Inc., Madison, WI): Pharmacia (Pharmacia, Inc., Piscataway, NJ): Qiagen (Qiagen, Chatsworth, CA): Showdex (Showa Denko America, Inc., New York, NY): Sigma (Sigma Chemical Co., St. Louis, MO): RIBI (RIBI Immunochemical Research Inc., Hamilton, MT); Accurate Chemical and Scientific Corp. (Accurate Chemical and Scientific Corp., Hicksville, NY): Kodak (Eastman-Kodak, Rochester, NY): and Stratagene (Stratagene, La Jolla, CA).

When a recombinant protein is described in the specification it is referred to in a short-hand manner by the amino acids in the toxin sequence present in the recombinant protein rounded to the nearest 10. The specification gives detailed construction details for all recombinant proteins such that one skilled in the art will know precisely which amino acids are present in a given recombinant protein.

The first set of Examples (Examples 1-5) was designed to develop an antidote to E. coli O157:H7 verotoxins and evaluate its effectiveness in vitro and in vivo. In the first experiments, high titer verotoxin antibodies were generated in laying hens hyperimmunized with chemically detoxified and/or native verotoxins. These Laying hens were immunized with either recombinant E. coli O157:H7 VT1 or VT2 (rVT1 and rVT2) treated with glutaraldehyde and mixed with adjuvant.

Next, toxin-reactive polyclonal antibodies were isolated by bulk fractionation from egg yolks pooled from hyperimmunized hens. Large quantities of polyclonal antibodies (IgY) were harvested from resulting eggs using a two-step polyethylene glycol fractionation procedure.

Third, the immunoreactivity and yields of VT IgY were analyzed by analytical immunochemical methods (e.g., enzyme immunoassay (EIA) and Western blotting). EIA and Western blot analysis showed that the resulting egg preparations contained high titer IgY that reacted with both the immunizing and the heterologous toxins (i.e., rVT1 IgY reacted against both rVT1 and rVT2, and vice versa).

Fourth. VT neutralization potency was analyzed *in vitro* using a Vero cytotoxicity assay. Vero cytotoxicity of rVT1 and rVT2 could be completely inhibited by VT IgY. These antibodies also demonstrated substantial verotoxin cross-neutralization.

Fifth, the efficacy of passively administered avian verotoxin antibodies in preventing the lethal effects of verotoxin poisoning was assessed in a mouse disease model. Toxin neutralizing antibodies were administered by parenteral dosing regimens to assess the most

effective strategy for therapeutic intervention. Efficacy of verotoxin antibodies was demonstrated using multiple murine disease models. In these experiments, antibodies prevented both the morbidity and lethality of homologous and heterologous toxins using a toxin/antitoxin premix format: mice infected orally with a lethal dose of viable *E. coli* O157:H7 were protected from both morbidity and lethality when treated parenterally four hours post-infection with either rVT1 or rVT2 antibodies; and mice given a lethal dose of *E. coli* O91:H21 (a particularly virulent strain which only produces VT2c, a VT2 structural variant) and treated parenterally up to 10 hours later with rVT1 IgY administered parenterally were protected from both morbidity and lethality.

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EXAMPLE 1

TOXIN ANALYSIS AND IMMUNIZATION

Purified recombinant *E. coli* O157:H7 verotoxins, rVT1 and rVT2, were obtained from Denka Sieken Co., Ltd. (Tokyo, Japan). Toxin genes were isolated, inserted into expression plasmids, and expressed in *E. coli*. Recombinant proteins were then purified by ammonium sulfate precipitation, ion exchange chromatography on DEAE Sephacryl and hydroxyapatite, and gel filtration chromatography by the supplier. Upon receipt, toxins were analyzed to verify identity, purity and toxicity, as described below.

20 A. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Samples of each toxin (2 μg) were heat-denatured in a buffer containing SDS and β-mercaptoethanol followed by electrophoresis on 10–20% gradient gels (Bio-Rad, Richmond, CA). Resolved polypeptide bands were visualized using the silver stain procedure of C.R. Merril, et al., "Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins." Science 211: 1437-1438 (1981).

VT1 and VT2 are each composed of subunit A and multiple copies of subunit B. Subunit A is often nicked into fragments A1 and A2 which are linked by a disulfide bridge. As shown in Figure 1, when separated by SDS-PAGE in the presence of β-mercaptoethanol. rVT1 resolved into 3 bands that corresponded to subunit A (~31 Kda), tragment A1 (~27 Kda) and a mixture of subunit B and fragment A2 (~4 Kda). Similarly, rVT2 resolved into subunit A (~33 Kda), fragment A1 (~27 Kda) and a mixture of subunit B and fragment A2 (~8 Kda) (Figure 1). In this Figure, rVT1 is in Lane 1, and rVT2 is in Lane 2; the positions of

molecular weight markers (Kda) are shown at the left. VT component polypeptides are identified at the right.

These results are consistent with previous reports of VT1 and VT2 purified from naturally occurring toxigenic strains (V. V. Padhye et al., "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From Escherichia coli O157:H7," Biochem. Biophys. Res. Commun., 139: 424-430 [1986]; and F. B. Kittel et al., "Characterization and inactivation of verotoxin 1 produced by Escherichia coli O157:H7," J. Agr. Food Chem., 39: 141-145 [1991]).

10 B. High Performance Liquid Chromatography (HPLC).

Chromatography was performed at room temperature (RT) under isocratic conditions using a Waters 510 HPLC pump. Eluted protein was measured using a Waters 490E programmable multi-wavelength detector (Millipore Corp., Milford, MA). The VT's were separated on an 8 x 300 mm (ID) Shodex KW803 column, using 10 mM sodium phosphate. 0.15 M NaCl. pH 7.4 (phosphate buffered saline [PBS]) as the mobile phase at a flow rate of 1 ml/min.

The purity of non-denatured rVT's was assessed by HPLC. As shown in the chromatographs in Figure 2, each toxin eluted at approximately 10 min. as a single absorbance peak at 280 nm. By integration of the area under each peak, the rVT's were shown to be >99% pure.

C. Vero Cell Cytotoxicity Assay.

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Cytotoxic activity of rVT1 and rVT2 was assessed using modified procedures of Padhye, et al. (V. V. Padhye et al., "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From Escherichia coli O157:H7." Biochem, Biophys, Res. Commun., 139: 424-430 [1986]), and McGee, et al., (Z. A. McGee, et al., "Local induction of tumor necrosis factor as molecular mechanism of mucosal damage by gonococci," Microbial Pathogenesis 12: 333-341 [1992]). Microtiter plates (96 well, Falcon, Microtest III) were inoculated with approximately 1 x 10⁴ Vero cells (ATCC, CCL81) per well (100 µl) and incubated overnight at 37°C in the presence of 5% CO₂ to form Vero cell monolayers, rVT1 and rVT2 solutions were serially diluted in Medium 199 supplemented with 5% fetal bovine serum (Life Technologies, Grand Island, NY), added to each well of the microtiter plates and incubated at 37°C for 18-24 hrs. Adherent (viable) cells were stained with 0.2% crystal

violet (Mallinckrodt) in 2% ethanol. Excess stain was rinsed away and the stained cells were solubilized by adding 100 µl of 1% SDS to each well. Absorbance of each well was measured at 570 nm. and the percent cytotoxicity of each test sample was calculated using the following formula:

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% Vero Cytotoxicity = [1 - (Absorbance Sample/Absorbance Control)] x 100

To determine whether the rVT's possessed potency equivalent to published cytotoxicity values, a Vero cell cytotoxicity assay was performed (Figure 3). Between 0.01-10.000 pg of either rVT1 or rVT2 was added to Vero cells. The amounts of rVT causing 50% cell death (CD₅₀), as calculated by second degree polynomial curve fitting were 0.97 pg and 1.5 pg, for rVT1 and rVT2, respectively. These results are consistent with CD_o values reported previously for naturally occurring VT1 and VT2 in the range 1-35 pg and 1-25 pg. respectively (M. Petric et al., Purification and biological properties of Escherichia coli verocytotoxin." FEMS Microbiol. Lett.. 41: 63-68 [1987]: V. L. Tesh, et al., "Comparison of relative toxicities of Shiga-Like toxins Type I and Type II for mice." Infect. Immun., 61: 3392-3402 [1993]: N. Dickie et al., "Purification of an Escherichia coli Serogroup O157:H7 verotoxin and its detection in North American hemorrhagic colitis isolates." J. Clin. Microbiol., 27: 1973-1978 [1989]; and U. Kongmuang, et al., "A simple method for purification of Shiga or Shiga-Like toxin from Shigella dysemeriae and Escherichia coli O157:H7 by immunoaffinity chromatography." FEMS Microbiol. Lett., 48: 379-383 [1987]). It has been observed that toxicity is lost with storage, explaining why higher amounts of toxin were used in the neutralization assays described below.

D. Mouse Lethal Dose Determination.

To verify rVT1 and rVT2 toxicity, male (20-22 g) CD-1 mice were injected intraperitoneally with varying amounts of rVT1 or rVT2 in 200 µL phosphate buffer. Doses were selected based on published LD₅₀ values for VT1 and VT2 in CD-1 mice. To minimize the sacrifice of live animals, a full statistical toxin LD₅₀ was not determined. Mice were observed for morbidity and mortality over 7-day period.

Further confirmation of rVT toxicity was obtained from mouse lethality experiments (Table 2). Mice were injected intraperitoneally with varying amounts of either rVT1 or rVT2 and observed 7 days for mortality. Within 72–120 hrs. post-injection, all of the mice died

from 100 ng of rVT1 or 10 ng of rVT2, respectively. This lethality study served as a verification of expected toxicity but not as a statistical determination of LD₅₀. Nonetheless, these results were consistent with toxicity studies which reported LD₅₀ values in CD-1 mice of 0.4–2.0 µg for purified VT1 and 0.001–1.0 µg for purified VT2 (V. L. Tesh. *et al.*, "Comparison of relative toxicities of Shiga-Like toxins Type 1 and Type II for mice." Infect. Immun., 61: 3392-3402 [1993]; and A. D. O'Brien, and G. D. LaVeck, "Purification and characterization of *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*," Infect. Immun. 40: 675-683 [1983]).

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Table 2. Lethality of rVT1 in CD-1 Mice

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ng VT1 Injected	Survivors/Total	Hours Post-Injection
	7/7	24 ± 2
100	5/7	48 ± 2
	0/7	72 ± 2
	7/7	24 ± 2
10	7/7	48 ± 2
	7/7	72 ± 2
	6/6	24 ± 2
1.0	6/6	48 ± 2
	6/6	72 ± 2

Table 3. Lethality of rVT2 in CD-1 Mice

ng VT2 Injected	Survivors/Total	
	3/6	Hours Post-Injection
10		48 ± 2
+	2/6	72 ± 2
	0/6	120 ± 2
}	5/6	48 ± 2
1.0	4/6	72 ± 2
	0/6	120 ± 2
0.1	6/6	48 ± 2
	6/6	72 ± 2
	6/6	120 ± 2

The recombinant toxins used in these studies thus appeared to contain protein components and toxicities consistent with literature reports for native toxins. Based on these structural and functional analyses, the rVT's were considered suitable as antigens to generate specific avian antibodies.

20 E. Antigen Preparation.

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Lyophilized samples, rVT1 and rVT2 were received and each was reconstituted with 2.5 mL of deionized water to a final concentration of 100 µg/ml in phosphate buffer. To form a toxoid, the solutions were then treated with 0.4% glutaraldehyde (Mallinckrodt) at 4°C overnight and stored at -20°C thereafter. When needed, toxoid was thawed and mixed 5:1 (volume:volume) with GERBU adjuvant (C. C. Biotech Corporation, Poway, CA). White Leghorn laying hens were injected subcutaneously with 25 µg of either rVT1 or rVT2 toxoid in adjuvant at 2-3 week intervals.

EXAMPLE 2 PEG EXTRACTION OF EGG YOLK ANTIBODY

Hyperimmune eggs were collected after 3 immunizations with toxoid. Egg yolks were separated from whites, pooled according to their immunogen group and blended with 4 volumes of 10 mM sodium phosphate. 150 mM NaCl. pH 7.4 (PBS). Polyethylene glycol

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8000 (PEG) (Amresco, Solon, OH) was then added to a final concentration of 3.5% and the mixture centrifuged at 10,000 x g for 10 min, to remove the precipitated lipid fraction, IgY-rich supernatant was filtered through cheesecloth and PEG was again added to a final concentration of 12%. The solution was centrifuged as above and the resulting supernatant discarded. The IgY pellet was then dissolved in PBS to either the original (1X PEG IgY) or 44 of the original (4X PEG IgY) yolk volume, filtered through a 0.45 μ membrane and stored at 4°C.

EXAMPLE 3

ANTITOXIN IMMUNOASSAYS

A. Enzyme Immunoassay (EIA).

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ElA was used to monitor antibody responses during the immunization course. Wells of 96-well Pro-Bind microtiter plates (Falcon, through Scientific Products, McGaw Park, IL) were each coated with 1 μg of rVT's (not toxoid) in PBS overnight at 2–8°C. Wells were washed 3 times with PBS containing 0.05% Tween-20 (PBS-T) to remove unbound antigen, and the remaining protein binding sites were blocked with PBS containing 1 mg/ml BSA for 60 min, at room temperature (RT), IgY, diluted in PBS, was then added to the wells and incubated for 1 hr, at 37°C. Wells were washed as before to remove unbound primary antibody and incubated for 1 hr, at 37°C with alkaline phosphatase-conjugated rabbit-antichicken IgG (Sigma Chemical Company, St. Louis, MO) diluted 1:1000 in PBS-T. Wells were again washed and 1 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Company, St. Louis, MO) in 50 mM Na₂CO₂ 10 mM MgCl₂ pH 9.5 was added and allowed to incubate at RT. Phosphatase activity was detected by absorbance at 410 nm using a Dynatech MR700 microtiter plate reader.

Laying Leghorn hens were immunized as described above (Example 1, part E), using glutaraldehyde-treated rVT's. Following several immunizations, eggs were collected and IgY harvested by PEG fractionation. Figures 4 and 5 show rVT1 or rVT2 specific antibody responses detected using EIA at dilutions of the original yolk IgY concentration of 1:30,000 and 1:6,000, respectively. IgY fractionated similarly from unimmunized hens (i.e., preimmune antibody) did not react with either antigen at test dilutions above 1:50. Although these EIA results indicate significant antibody responses, prior experience with other toxin antigens has shown that optimization of immunization regimens, including increasing the amount of

antigen, can yield titers in excess of 1:100.000 (B. S. Thalley, et al., "Development of an Avian Antitoxin to Type A Botulinum Neurotoxin." in Botulinum and Tetanus Neurotoxins:

Neurotransmission and Biomedical Aspects. B. R. DasGupta, (ed.) [Plenum Press, New York, 1993] pp. 467-472). As may be expected due to their structural homology and consistent with previous reports (e.g., V. V. Padhye et al., "Production and characterization of monoclonal antibodies to verotoxins 1 and 2 from Escherichia coli O157:H7." J. Agr. Food Chem., 39: 141-145 [1989]: S. C. Head et al., "Purification and characterization of verocytotoxin 2."

FEMS Microbiol, Lett., 51: 211-216 [1988]; and N. C. Strockbine et al., "Characterization of Monoclonal Antibodies against Shiga-Like Toxin from Escherichia coli," Infect. Immun., 50: 695-700 [1985]). Figures 4 and 5 also demonstrate that antibodies generated against one toxin cross-reacted in vitro with the other toxin.

B. Western Blot Analysis.

Western blots (Figure 6) performed to determine the reactivity of rVT antibodies against constituent VT polypeptides showed that rVT1 and rVT2 antibodies reacted with subunit A and fragment A1 of either toxin, and with subunit B and fragment A2 of rVT1 only. In this Figure, Panel A contains preimmune IgY, Panel B contains rVT1 IgY, and Panel C contains rVT2 IgY. Lane 1 in each panel contains rVT1 (2µg) and Lane 2 contains rVT2 (2 µg). Preimmune IgY was largely nonreactive to either rVT. Both rVT IgY preparations, however, failed to react with subunit B and fragment A2 of rVT2. Some explanations for this lack of measurable reactivity might include poor immunogenicity, denaturation of the immunogen during glutaraldehyde treatment, loss of conformational epitopes due to detergent or reducing agent, or poor transfer to nitrocellulose.

To resolve the high and low molecular weight components. 2 µg each of rVT1 and rVT2 were separated by SDS-PAGE (described above) and then transferred to nitrocellulose paper using the Milliblot-SDE system (Millipore, Medford, MA) according to the manufacturer's instructions. Paper strips were stained temporarily with Ponceau S (Sigma Chemical Company, St. Louis, MO) to visualize the polypeptides and then blocked overnight in PBS containing 5% dry milk. Each strip was agitated gently in IgV diluted in PBS-T for 2 hrs. at RT. Strips were each washed with three changes of PBS-T to remove unbound primary antibody and incubated for 2 hrs. at RT with goat anti-chicken alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, MD) diluted 1:500 in PBS-T containing 1 mg/ml BSA. The blots were washed as before and rinsed in 50 mM Na₂CO₃, pH 9.5. Strips were

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submerged in alkaline-phosphatase substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (Kirkegaard and Perry) until sufficient signal was observed. Color development was stopped by flooding the blots with water.

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EXAMPLE 4

IN VITRO TOXIN NEUTRALIZATION: VERO CELL ASSAY

IgY neutralization of rVT1 and rVT2 was assessed using the modified Vero cytotoxicity assay described above (Example 1, part C). Various concentrations of IgY, diluted in Medium 199 supplemented with 5% fetal bovine serum (GIBCO), were mixed with sufficient toxin to cause 50% cell death and allowed to incubate at 37°C for 60 minutes. These toxin/antibody mixtures were then added to Vero cell-coated microtiter plate wells according to the procedure described above (Example 1, part C).

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The toxin neutralization capacity of the rVT antibodies was analyzed first using a Vero cell toxicity assay. The results in Figure 7 show that rVT1 IgY neutralized completely the cytotoxic activity of rVT1 at an endpoint dilution of 1/320. Furthermore, rVT2 IgY neutralized the heterologous rVT1 toxin, but at a higher endpoint concentration.

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In a similar experiment (see Figure 8), rVT1 and rVT2 antibodies were each able to neutralize rVT2 at equivalent endpoint dilutions. This strong cross-neutralization correlates with the observed strong cross-reactivity of VT1 IgV with VT2 A seen on Western blots (Figure 6). These results show that IgV antibodies are able to neutralize effectively VT cytotoxicity and that the antibodies can cross-neutralize structurally-related heterologous toxins.

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EXAMPLE 5

TOXIN NEUTRALIZATION: MOUSE ASSAYS

A. Toxin Challenge Model.

IgY in PBS was premixed with a lethal dose of toxin (as determined above) and injected intraperitoneally into male CD-1 (20-22 gm) mice. Mice were observed for a 7-day period for signs of intoxication such as ruffled fur, huddling and disinclination to move, followed by hind leg paralysis, rapid breathing and death. Untreated, infected mice usually died within 12 hrs. after signs of severe illness (i.e., within 48-72 hrs. post-injection).

Once it was demonstrated that rVT antibodies were able to neutralize rVT cytotoxicity in vitro, protection experiments were next performed in mice. First, animals were challenged with rVT premixed with rVT IgY to determine whether toxin lethality could be neutralized under conditions optimal for antigen/antibody reaction. Tables 4 and 5 show that antibodies premixed with the homologous toxin (e.g., rVT1 with rVT1 IgY) prevented lethality of rVT. Preimmune IgY was unable to neutralize either toxin in these studies.

Table 4
Neutralization of rVT1 Using rVT IgY

100 ng rVT2 Premixed*	Survivors/Total	n
Preimmune Antibody	0/12	<i>p</i>
rVT1 Antibody	12/12	< 0.001
rVT2 Antibody	12/12	< 0.001

*Toxin was pre-mixed with IgY and incubated for 1 hour at room temperature prior to administration.

Table 5

Neutralization of rVT2 Using rVT IgY

10 ng rVT1 Premixed*	Survivors/Total	p
Preimmune Antibody	0/12	<i>r</i>
rVT1 Antibody	12/12	< 0.001
rVT2 Antibody	12/12	< 0.001

*Toxin was pre-mixed with IgY and incubated for 1 hour at room temperature prior to administration.

Antibodies premixed with the heterologous toxin (e.g., rVT2 with rVT1 IgY) also prevented lethality in vivo. These data are in contrast to previous observations where rabbit polyclonal antibodies generated against either toxin were cross-reactive with the heterologous toxin by EIA and Western blot, but were unable to neutralize the heterologous toxin in either Vero cell cytotoxicity and mouse lethality assays (S. C. Head, et al., "Serological differences between verocytotoxin 2 and Shiga-like toxin II," Lancet ii: 751 [1988]: S. C. Head et al., "Purification and characterization of verocytotoxin 2." FEMS Microbiol, Lett., 51: 211-216

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[1988]: N. C. Strockbine et al.: "Characterization of Monoclonal Antibodies against Shiga-Like Toxin from Escherichia coli." Infect. Immun.: 50: 695-700 [1985]: and V. V. Padhye et al.: "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From Escherichia coli O157:H7." Biochem. Biophys. Res. Commun.: 139: 424-430 [1986]).

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However, Head *et al.*, showed that VT2 B-subunit specific monoclonal antibodies neutralized VT1 weakly in a Vero cytotoxicity assay (S. C. Head, *et al.*, "Serological differences between verocytotoxin 2 and Shiga-like toxin II." Lancet ii: 751 [1988]). In a report by Donohue-Rolfe, *et al.*, a VT2 B subunit-specific monoclonal antibody neutralized both VT1 and VT2 completely in a Hela cytotoxicity assay (A. Donohue-Rolfe *et al.*, "Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross reactive monoclonal antibodies." Infect. Immun., 57: 3888-3893 [1989]).

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These results showed for the first time complete cross-neutralization in Vero cell cytotoxicity and mouse lethality assays, revealing that VT1 and VT2 do indeed share common neutralizing epitopes. These results may indicate that hens generate different antibody specificities as compared to mammals, and/or that differences in immunization methods might have maintained the immunogenicity of conformational epitopes necessary for cross-neutralization. Nonetheless, this cross-neutralization suggests that IgY antibodies may contain the range of reactivities essential for an effective antitoxin.

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B. Viable organism infection model.

Streptomycin-resistant *E. coli* O157:H7 (strain 933 cu-rev) or *E. coli* O91:H21 (strain B2I 1) (both kindly provided by Dr. Alison O'Brien, Dept. of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD) were used in a murine infection model described by Wadolkowski, *et al.* (E. A. Wadolkowski *et al.*, "Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7," Infect. Immun., 58: 2438-2445 [1990]). Organisms were grown in Luria broth and incubated overnight at 37°C in an Environ Shaker (Lab Line, Melrose Park, IL) (T. Maniatis *et al.*, Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., [1982]). Bacterial suspensions were centrifuged at 6700 x g for 5 minutes. The resulting pellet was then washed twice with sterile PBS and resuspended in sterile 20% (w/v) sucrose. Five to 8 week-old male CD-1 mice were provided drinking water containing 5 mg/ml streptomycin sulfate *ad libitum* for 24 hrs. Food and water were then withheld for

another 16–18 hrs. after which mice were challenged orally with 10th streptomycin-resistant *E. coli* O157:H7 or O91:H21. Mice were housed individually and permitted food and water containing 5 mg/ml streptomycin sulfate. IgY was injected intraperitoneally at varying times post-infection and animals observed for both morbidity and mortality for 10 days.

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To monitor bacterial colonization in animals. I gram of feces was collected. homogenized, and plated onto MacConkey agar medium (Difco Laboratories, Detroit, MI) containing 100 µg/ml streptomycin and incubated at 37°C as described by Wadolkowski, et al. (E. A. Wadolkowski et al., "Mouse model for colonization and disease caused by enterohemorrhagic Escherichia coli O157:H7," Infect. Immun., 58: 2438-2445 [1990]). The serotype of E. coli O157:H7, 933 cu-rev excreted in feces was confirmed by slide agglutination with O- and H-specific antisera (Difco Laboratories, Detroit, MI).

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Kidneys were removed from experimental animals and fixed in 10% buffered neutral formalin. Sections of parafilm-embedded tissue were stained with hematoxylin and eosin (General Medical Laboratories, Madison, WI) and examined by light microscopy. All tissue sections were coded to avoid bias before microscopic examination to determine renal pathology.

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The toxin neutralization ability of rVT IgY was further studied using a streptomycintreated CD-1 mouse infection model. This model was chosen because it produces definitive systemic pathology and reproducible mortality.

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In contrast to previous studies by Wadolkowski. et al. (E. A. Wadolkowski et al...
"Acute renal tubular necrosis and death of mice orally infected with Escherichia coli strains that produce Shiga-like toxin Type II." Infect. Immun.. 58: 3959-3965 [1990]), where mice were given subunit-specific monoclonal antibodies prior to infection, the mice in this study were inoculated orally with 2 x 10¹⁰ viable E. coli O157:H7 (strain 933 cu-rev) and treated with rVT IgY 4 hrs. following inoculation. Fecal cultures showed that 10⁷-10⁸ challenge organisms per gram of feces were shed throughout the course of the experiment, thus confirming that infection was established. Tables 6 and 7 show that animals treated with either rVT1 or rVT2 IgY were protected from lethality caused by infection (p<0.01 and p<0.001, respectively) and that preimmune IgY failed to provide protection to the mice.

Table 6
Protection of Mice From E. coli O157:H7
With rVT1 IgY

IgY Treatment	Survivors/Total	p	Morbidity/Total
Preimmune Antibody	0/5		5/5
rVT1 Antibody	9/10	< 0.01	1/10

^{*}lgY was administered intraperitoneally 4 hours following infection, and once daily for 10 days thereafter.

Table 7
Protection of Mice From E. coli O157:H7
With rVT2 IgY

IgY Treatment	Survivors/Total	p	Morbidity/Total
Preimmune Antibody	0/6		6/6
rVT2 Antibody	10/10	< 0.005	0/10

*lgY was administered intraperitoneally 4 hours following infection, and once daily for 10 days thereafter.

Renal histopathology (see Figure 9) of the control (preimmune IgY) animals showed dilation, degeneration and renal tubular necrosis with no glomerular damage. This is consistent with previous reports showing that renal tubular involvement occurs predominantly in this streptomycin-treated mouse infectivity model (E. A. Wadolkowski et al., "Acute renal tubular necrosis and death of mice orally infected with Escherichia coli strains that produce Shiga-like toxin Type II." Infect. Immun., 58: 3959-3965 [1990]). Importantly, none of the survivors exhibited similar signs of morbidity though treated with IgY 4 hrs. after infection (see Figure 9).

Furthermore, avian antibodies generated against rVT1 were able to prevent both mortality and morbidity in a mouse model where VT2 alone is implicated in the pathogenesis and lethality of *E. coli* O157:H7 strain 933 cu-rev (E. A. Wadolkowski *et al.*. "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type 11." Infect. Immun., 58: 3959-3965 [1990]).

To assess the broader utility of the IgY verotoxin antibodies in treating VTEC-associated disease, the mouse infectivity study was performed using a more virulent VTEC serotype known to produce VT2c—a structural variant of VT2—but not VT1 (S. W. Lindgren

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et al., "Virulence of enterohemorrhagic Escherichia coli O91:H21 clinical isolates in an orally infected mouse model." Infect. Immun., 61: 3832-3842 [1993]).

Mice were inoculated orally with 5 x 10° E. coli. O91:H21 (strain B2F1) and treated subsequently with IgY. Notably, the heterologous rVT1 IgY protected strongly against the lethal effects of the VT2c structural variant, even when administered as long as 10 hrs. following infection (Table 8). Ten hours was the longest treatment window tested in this study. Only 1 of the 8 animals treated with rVT1 IgY died (p <0.02), and those that survived showed no overt signs of renal histopathology (i.e., acute bilateral tubular necrosis). It can thus be concluded that rVT1 IgY completely neutralized toxicity of VT2c, indicating its potential as a therapeutic for at least one other pathogenic VTEC.

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Table 8
Protection of Mice From E. coli (191:H21)
With rVT1 lgY

IgY Treatment	Survivors/Total	p	Morbidity/Total
Preimmune Antibody	0/7		7/7
rVT1 Antibody	7/8	< 0.02	1/8

*IgY was administered intraperitoneally 10 hours following infection, and once daily for 8 days thereafter.

These Examples highlight several important findings supporting the feasibility of using verotoxin antitoxin. First, polyclonal IgY generated against either VT1 or VT2 from E. coli O157:H7, cross-reacted with and fully cross-neutralized the toxicity of the non-immunizing toxin both in vitro and in vivo. Second, recombinant toxins fully neutralized the toxicity of naturally-occurring toxins produced by E. coli O157:H7 during the course of infection. Third, antibodies generated against rVT1 from E. coli O157:H7 could prevent morbidity and mortality in mice infected orally with lethal doses of E. coli O91:H21, a particularly virulent strain which only produces VT2c, suggesting their utility in preventing systemic sequelae. Because VT1 is identical to Shiga-toxin (A. D. O'Brien et al., "Shiga and Shiga-like toxins. Microbial Rev., 51: 206-220 [1987]), VT antibodies may also be useful in preventing complications stemming from Shigella dysenteriae infection. Finally, animals treated with VT

IgY were protected against both death and kidney damage when treated as long as 10 hrs. after infection, supporting the hypothesis that a window for antitoxin intervention exists.

These studies strongly support the use of parenterally-administered, toxin-specific IgY as a antitoxin to prevent life-threatening complications associated with *E. voli* O157:H7 and other VTEC infections. It is contemplated that this approach would be most useful in preventing HUS and other complications when administered after the onset of bloody diarrhea and before the presentation of systemic disease.

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The VT IgY developed in these studies were shown to react with and neutralize both recombinant and naturally-occurring VT. The antibody titers as measured by EIA are indicative of reasonable antibody production in the hen, however much higher production levels can be obtained with larger immunizing doses.

The results from these Examples clearly demonstrate the feasibility and provide the experimental basis for development of an avian antidote for E. coli O157:H7 verotoxins suitable for use in humans. In contrast to previous reports showing that rabbit polyclonal VT1 and VT2 antibodies cross-reacted, but did not cross-neutralize the heterologous toxin in Vero cytotoxicity or in mouse lethality studies (e.g., V. V. Padhye et al., "Production and characterization of monoclonal antibodies to verotoxins 1 and 2 from Escherichia coli O157:H7." J. Agr. Food Chem., 39: 141-145 [1989]; S. C. Head et al., "Purification and characterization of verocytotoxin 2." FEMS Microbiol. Lett., 51: 211-216 [1988]: and N. C. Strockbine et al.. "Characterization of monoclonal antibodies against Shiga-like toxin from Escherichia coli." Infect. Immun., 50: 695-700 [1985]), these data provide the first demonstration of cross-neutralization in vivo. Antibodies against one toxin neutralized completely the heterologous toxin in both Vero cytotoxicity and mouse lethality assays. Both rVT1 and rVT2 antibodies also prevented morbidity (as assessed by renal histopathology) and mortality in mice infected with lethal doses of E. coli O157:H7 - the etiologic agent in 90% of the documented cases of hemolytic uremic syndrome (HUS) in the U.S. (P. M. Griffin and R. V. Tauxe. "The epidemiology of infections caused by Escherichia coli O157:H7, other enterohemorrhagic E. coli, and the associated hemolytic uremic syndrome." Epidemiol. Rev., 13: 60 [1990]). With at least two other VTEC serotypes known to cause HUS, the finding that rVT1 antibodies neutralized a VT2 variant produced by E. coli O91:H21 suggests that avian polyclonal antibodies may provide an effective antidote against other verotoxinproducing E. coli. These data also show for the first time, that antibodies may be administered after infection and still protect against morbidity and mortality.

EXAMPLE 6 EXPRESSION OF TOXIN GENES

The previous Examples clearly showed that avian polyclonal antibodies to recombinant toxins protected animals infected with verotoxigenic *E. coli*. This Example includes expression of toxin genes (A and B subunits alone and together as whole toxins) in suitable prokaryotic expression systems to achieve high levels of VT antigen production.

The sequence of the toxin gene has been determined (see e.g., M.P. Jackson et al., "Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from Escherichia coli 933." 44:109 [1987]). The coding regions of the A and B subunits of VT-1 are listed in SEQ ID NOS:1 and 3, respectively. The corresponding amino acid sequence of the A and B subunits of the VT-1 toxin are listed in SEQ ID NOS:2 and 4, respectively. The coding regions of the A and B subunits of VT-2 are listed in SEQ ID NOS:5 and 7, respectively. The corresponding amino acid sequence of the A and B subunits of the VT-2 toxin are listed in SEQ ID NOS:6 and 8, respectively. In addition, SEQ ID NOS:9 and 10 list the sequences which direct the expression of a poly-cistronic RNA capable of directing the translation of both the A and B subunits from the VT-1 and VT-2 genes, respectively.

In choosing a strategy for recombinant VT antigen production, there are three primary technical factors to consider. First, the appropriate VT antigen components representing the spectrum of toxin epitopes encountered in nature must be utilized. Second, the protein antigens must be expressed at sufficient levels and purity to enable immunization and large-scale antibody purification. Third, the neutralizing epitopes must be preserved in the immunogen and immunoabsorbant. Approaches that offer the greatest promise for high level expression of periplasmically localized, native, affinity-tagged proteins were developed. Figure 10 shows the fusion constructs of VT components and affinity tags.

A. Expression of affinity-tagged C-terminal constructs.

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The VT1 and VT2 A and B subunits (SEQ ID NOS:1, 3, 5 and 7) are cloned into the pET-23b vector (Novagen). This vector is designed to allow expression of native proteins containing C-terminal poly-His tags. The vector utilizes a strong T7 polymerase promoter to drive high level expression of target proteins. The methionine initiation codon is engineered to contain a unique Ndel restriction enzyme site (CATATG). The VT1 and VT2 genes are engineered to convert the signal sequence methionine codon into a Ndel site utilizing PCR

mutagenesis. PCR primers were designed which contain the sequence GCCAT fused to the first 20-24 bases of the genes (starting at the ATG start codon of the signal tag: SEQ ID NOS:12-19, see Table below). Upon PCR amplification, the 5' start codon of each gene is converted to an NdeI site, compatible with the pET-23 vector-encoded NdeI site, allowing cloning of the amplified genes into the vector without the addition of vector-encoded amino acids.

Primers containing the C-terminal 7 codons of each gene (21 bases) fused to the sequence CTCGAGCC were synthesized, in order to add a C-terminal poly-His tag to each gene. The underlined bases are an XhoI site, that is compatible with the XhoI site of the pET-23 vector. These primers precisely delete the native stop codons, and when cloned into the pET-23 vector, add a C-terminal extension of "LeuGluHisHisHisHisHisHisHis" (SEQ ID NO: 11). The following table lists the primer pairs are utilized to create PCR fragments containing the A and B subunits derived from VT-1 and VT-2 toxin genes suitable for insertion into the pET-23b vector.

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Table 9
Primers

Toxin Gene and Subunit	N-terminal Primer	C-terminal Primer
VT-1 Subunit A	SEQ ID NO:12	SEQ ID NO:13
VT-1 Subunit B	SEQ ID NO:14	SEQ ID NO:15
VT-2 Subunit A	SEQ ID NO:16	SEQ ID NO:17
VT-2 Subunit B	SEQ ID NO:18	SEQ ID NO:19
VT-1 Subunits A and B	SEQ ID NO:12	SEQ ID NO:15
VT-2 Subunits A and B	SEQ ID NO:16	SEQ ID NO:19

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Thus, utilizing PCR amplification with the above modified N- and C-terminal primers, the A and B subunits of VT1 and VT2 are expressed as proteins containing an 8 amino acid C-terminal extension bearing an poly-histidine affinity tag. The amino acid sequence of the histidine-tagged VT-1 A subunit produced by expression from the pET-23b vector is listed in SEQ ID NO:21 (the associated DNA sequence is listed in SEQ ID NO:20): the amino acid sequence of the histidine-tagged VT-1 B subunit is listed in SEQ ID NO:23 (the associated

DNA sequence is listed in SEQ ID NO:22): the amino acid sequence of the histidine-tagged VT-2 A subunit is listed in SEQ ID NO:25 (the associated DNA sequence is listed in SEQ ID NO:24): the amino acid sequence of the histidine-tagged VT-2 B subunit is listed in SEQ ID NO:27 (the associated DNA sequence is listed in SEQ ID NO:26).

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Both subunits may be expressed from a single expression constructs by utilizing SEQ ID NOS:12 and 15 to prime synthesis of the VT-1 toxin gene and SEQ ID NOS:16 and 19 to prime synthesis of the VT-2 toxin gene. The resulting PCR products are cleaved with NdeI and NhoI. as described for the cloning of the subunit genes into the pET-23b vector. Expression of the A and B subunits from such an expression vector, results in the expression of a native A subunit and a his-tagged B subunit. As the A and B subunits assemble into a complex, the presence of the his-tag on only the B subunit is sufficient to allow purification of the holotoxin on metal chelate columns as described below.

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The proofreading *Pfii* polymerase (Stratagene) is utilized for PCR amplification to reduce the error rate during amplification. Genomic DNA from an *E. coli* O157:H7 strain is utilized as template DNA. Following the PCR, the amplification products are digested with *Ndel* and *Xhol* and cloned into the pCR-Script SK cloning vehicle (Stratagene) to permit DNA sequence analysis of the amplified products. The DNA sequence analysis is performed to ensure that no base changes are introduced during amplification. Once the desired clones are identified by DNA sequencing, the inserts are then excised utilizing *Ndel* and *Xhol*, and cloned into a similarly cut pET-23b vector to create the expression constructs. According to the published sequences, neither the VT1 nor VT2 genes contain either of these restriction sites.

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The poly-His-tagged proteins produced by expression of the VT-1 and VT-2 gene sequences in the pET-23b constructs are then purified by IMAC. This method uses metal-chelate affinity chromatography to purify native or denatured proteins which have histidine tails (see e.g., K. J. Petty, "Metal-Chelate Affinity Chromatography," in Current Protocols in Molecular Biology, Supplement 24, Unit 10.11B [1993]).

B. Expression of Toxin Containing N-terminal Affinity Tags

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Two expression systems, pMal-p2 and pFLAG-1 are utilized to attach an N-terminal affinity tag to the A subunits from the VT-1 and VT-2 toxins.

MBP-tagged constructs. To construct A chains containing the maltose binding protein (MBP) at the N-terminus of the A subunit. PCR amplified gene products are cloned into the

pMal-p2 vector (New England Biolabs) as C-terminal fusions to a periplasmically-secreted version of the MBP. The MBP selectively binds to amylose resins and serves as an affinity tag on the MBP/A subunit fusion protein. The pMal-p2 vector contains an engineered factor Xa cleavage site, which permits the removal of the affinity tag (i.e., MBP) from the fusion protein after purification.

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The MBP/A subunit fusions are generated as follows. The VT1 and VT2 A subunits are PCR-amplified utilizing the following DNA primers. SEQ ID NOS:28-31: SEQ ID NOS:28 and 29 comprise the 5' and 3' primers, respectively, for the amplification of the VT1 A subunit: SEQ ID NOS:30 and 31 comprise the 5' and 3' primers, respectively, for the amplification of the VT2 A subunit. In both cases, the 5' or N-terminal primer contains the sequence CGGAATTC fused to the first codon of the mature polypeptide (rather than the start of the signal peptide, since the MBP signal peptide is utilized). These 5' primers contain an engineered *EcoRI* site that is not contained internally in either gene, that is compatible with the *EcoRI* site of the pMal-p2 vector. The 3' or C-terminal primers incorporate an *MhoI* site as described above for the generation of the His-tagged toxins, but in this case, the 3' primer is designed to include the natural termination codon of the A subunits.

The genes are amplified, cloned into pCR-Script SK, and sequenced as described above. The inserts are then excised with *EcoRI* and *XhoI*, and cloned into *EcoRI/SalI*-cleaved pMal-p2 vector (*SalI* and *XhoI* sites are compatible). This construct allows expression and secretion of the VT1 and VT2 A subunit genes as C-terminal fusions with MBP. The amino acid sequence of the MBP/VT-1A fusion protein is listed in SEQ ID NO:33 (the associated DNA sequence is listed in SEQ ID NO:32). The amino acid sequence of the MBP/VT-2A fusion protein is listed in SEQ ID NO:35 (the associated DNA sequence is listed in SEQ ID NO:34).

The resulting fusion proteins are then affinity purified on an amylose column and the bound fusion protein is eluted under mild conditions by competition with maltose. The MBP N-terminal-tagged A subunits are cleaved with factor Xa and the MBP is removed by chromatography on an amylose column. The resulting A subunits which contain a 4 amino acid N-terminal extension are then used as immunogens.

Flag tag constructs. In an alternative embodiment, the VT1 and VT2 A subunit genes are engineered to contain the "flag tag" through the use of the pFLAG-1 vector system. The flag tag is located between the OmpA secretion signal sequence and the authentic N-

terminus of the target protein in the pFlag-1 vector. To construct N-terminal flag-tagged A chains, the *EcoRI/Xhol* A subunit PCR fragments (generated as described above for the MBP fusion proteins) are cloned into identically cleaved pFlag-1 vector (Eastman-Kodak), to produce an expression construct utilizing the *OmpA* signal peptide for secretion of A subunit fusion proteins containing the flag peptide at the N-terminus. After secretion, the periplasmic protein contains the N-terminal 8 amino acid flag tag, followed by 4 vector-encoded amino acids fused to the recombinant A subunit. The amino acid sequence of the flag tag/VT-1 A subunit fusion protein is listed in SEQ ID NO:37 (the associated DNA sequence is listed in SEQ ID NO:36). The amino acid sequence of the flag tag/VT-2 A subunit fusion protein is listed in SEQ ID NO:39 (the associated DNA sequence is listed in SEQ ID NO:38).

The flag tag fusion proteins are then purified by immunoaffinity chromatography utilizing a calcium-dependent monoclonal antibody (Antiflag M1: Eastman-Kodak). Mild elution of purified protein is achieved by chelating the calcium in the column buffer with ethylenediamine tetraacetic acid (EDTA).

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C. Evaluation of fusion construct expression.

The fusion constructs described above are expressed in *E. coli* strain BL21, or T7 polymerase-containing derivatives [e.g., BL21(DE3), BL21(DE3) pLysS, BL21(DE3)pLysE] (Novagen) for pET plasmids, and periplasmically-secreted recombinant protein purified by affinity chromatography. Recombinant proteins are analyzed for correct conformation by testing the following parameters:

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a) It is believed that the B subunit must associate into pentamers to be conformationally correct. This is assessed by reducing and native SDS-PAGE analyses of native and chemically-cross-linked proteins and sizing HPLC:

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b) It is believed that a properly folded A subunit is expected to retain its native enzymatic activity. This is tested by its capacity to inhibit protein synthesis in an *in vitro* toxicity assay;

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c) It is believed that in vitro toxicity of assembled recombinant holotoxin is compared to commercially available holotoxins to determine whether recombinant A and B subunits can assemble into functional holotoxin. The

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purified N-terminal-tagged A subunits (after cleavage and purification from MBP or untreated flag-tagged proteins) are combined *in vitro* with the corresponding B chains, and their toxicity evaluated utilizing a quantitative microtiter cytotoxicity assay, such as that described by M.K. Gentry and M. Dalrymple, "Quantitative Microtiter Cytotoxicity Assay for *Shigella* Toxin," J. Clin. Microbiol., 12:361-366 (1980).

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
 - (i) APPLICANT: OPHIDIAN PHARMACEUTICALS, INC.
 - (ii) TITLE OF INVENTION: TREATMENT FOR VEROTOXIN-PRODUCING E. COLI
 - (iii) NUMBER OF SEQUENCES: 39
 - (iv) CORRESPONDENCE ADDRESS:
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 - (B) STREET: 220 MONTGOMERY STREET, SUITE 2200
 - (C) CITY: SAN FRANCISCO
 - (D) STATE: CALIFORNIA
 - (E) COUNTRY: UNITED STATES OF AMERICA
 - (F) ZIP: 94104
 - (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible

 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: Patentin Release #1.0, Version #1.25
 - (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
 - (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: CARROLL, PETER G.
 - (B) REGISTRATION NUMBER: 32,837
 - (C) REFERENCE/DOCKET NUMBER: OPHD-02171
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- (2) INFORMATION FOR SEQ ID NO:1:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 945 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..945
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- ATG AAA ATA ATT ATT TTT AGA GTG CTA ACT TTT TTC TTT GTT ATC TTT Met Lys Ile Ile Phe Arg Val Leu Thr Phe Phe Val Ile Phe
- TCA GTT AAT GTG GTG GCG AAG GAA TTT ACC TTA GAC TTC TCG ACT GCA Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala 96 25
- AAG ACG TAT GTA GAT TCG CTG AAT GTC ATT CGC TCT GCA ATA GGT ACT Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr
- CCA TTA CAG ACT ATT TCA TCA GGA GGT ACG TCT TTA CTG ATG ATT GAT Pro Leu Gln Thr lle Ser Ser Gly Gly Thr Ser Leu Leu Net lle Asp 192 €0

WO 96/30043 PCT/US96/04c >

AGT Ser 65	. G1 A	TCA Ser	GGG Gly	GAI Asp	AAT Asn 70	Leu	TTT	GCA Ala	GTI Val	GAT Asp 75	Va]	C AGA l Arg	GGG Gly	ATA	GAT Asp 80	240
GCA Ala	GAG Glu	GAA Glu	GGG Gly	CGG Arg 85	Phe	AAT Asn	AAT Asn	CTA Leu	CGG Arg	Leu	ATT	GTT Val	GAA Glu	CGA Arg	AAT Asn	288
AAT Asn	TTA Leu	TAT Tyr	GTG Val 100	ACA Thr	GGA Gly	TTT Phe	GTT Val	AAC Asn 105	Arg	ACA Thr	AAT Asn	TAAT ASD	GTT Val 110	Phe	TAT	336
CGC Arg	TTT Phe	GCT Ala 115	Asp	TTT Phe	TCA Ser	CAT His	GTT Val 120	Thr	TTT Phe	CCA Pro	GGT Gly	ACA Thr 125	ACA Thr	GCG Ala	GTT Val	384
ACA Thr	TTG Leu 130	TCT Ser	GGT Gly	GAC Asp	AGT Ser	AGC Ser 135	TAT Tyr	ACC Thr	ACG Thr	TTA Leu	CAG Gln 140	CGT Arg	GTT Val	GCA Ala	GGG Gly	432
ATC Ile 145	AGT Ser	CGT Arg	ACG Thr	GGG Gly	ATG Met 150	CAG Gln	ATA Ile	AAT Asn	CGC Arg	CAT His 155	TCG Ser	TTG Leu	ACT Thr	ACT Thr	TCT Ser 160	480
TAT Tyr	CTG Leu	GAT Asp	TTA Leu	ATG Met 165	TCG Ser	CAT His	AGT Ser	GGA Gly	ACC Thr 170	TCA Ser	CTG Leu	ACG Thr	CAG Gln	TCT Ser 175	GT3 Val	526
GCA Ala	AGA Arg	GCG Ala	ATG Met 180	TTA Leu	CGG Arg	TTT Phe	GTT Val	ACT Thr 185	GTG Val	ACA Thr	GCT Ala	GAA Glu	GCT Ala 190	TTA Leu	CGT Arg	576
TTT Phe	CGG Arg	CAA Gln 195	ATA Ile	CAG Gln	AGG Arg	GGA Gly	TTT Phe 200	CGT Arg	ACA Thr	ACA Thr	CTG Leu	GAT Asp 205	GAT Asp	CTC Leu	AGT Ser	624
GGG Gly	CGT Arg 210	TCT Ser	TAT Tyr	GTA Val	ATG Met	ACT Thr 215	GCT Ala	GAA Glu	GAT Asp	GTT Val	GAT Asp 220	CTT Leu	ACA Thr	TTG Leu	AAC Asn	672
TGG Trp 225	GGA Gly	AGG Arg	TTG Leu	AGT Ser	AGC Ser 230	GTC Val	CTG Leu	CCT Pro	GAC Asp	TAT Tyr 235	CAT His	GGA Gly	CAA Gln	GAC Asp	TCT Ser 240	720
GTT Val	CGT Arg	GTA Val	GSA Gl;	AGA Arg 245	ATT Ile	TCT Ser	TTT Phe	GGA Gly	AGC Ser 250	ATT Ile	AAT Asn	GCA Ala	Ile	CTG Leu 255	GGA Gly	768
AGC Sei	GTG Val	GCA Ala	TTA Leu 260	ATA Ile	CTG Leu	AAT Asn	TGT Cys	CAT His 265	CAT His	CAT His	GCA Ala	TCG Ser	CGA Arg 270	GTT Val	GCC Ala	816
AGA Arg	ATG Met	GCA Ala 275	TCT Ser	GAT Asp	GAG Glu	Phe	CCT Pro 280	TCT Ser	ATG Met	TGT Cys	CCG Pro	GCA Ala 285	GAT Asp	GGA Gly	AGA Arg	864
Val	CGT Arg 290	GGG Gly	ATT Ile	ACG Thr	His	AAT Asn 295	AAA Lys	ATA Ile	TTG Leu	TGG Trp	GAT Asp 300	TCA Ser	TCC . Ser	ACT Thr	CTG Leu	912
GGG Gly 305	GCA Ala	ATT Ile	CTG . Leu !	Met	CGC . Arg . 310	AGA Arg	ACT Thr	ATT Ile	AGC Ser	AGT Ser 315						945

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 315 amino acids (B) TYPE: amino acid

 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
- Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe
- Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala
- Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr
- Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp
- Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp
- Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn
- Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr
- Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val 120
- Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly 135
- Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser 150
- Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val
- Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg 185
- Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser
- Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn 210
- Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser 235 230 235
- Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly
- Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala 265
- Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg 280
- Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu 295
- Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser 310

SENTON SINO GERMANA I .

(2) INFORMATION FOR SEQ ID NO:3: (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 267 base pairs
- (B) TYPE: nucleic acid (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..267
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATO Met	AAA Lys	AAA Lys	ACA Thr	TTA Leu 5	TTA Leu	ATA Ile	GCT Ala	GCA Ala	TCG Ser 10	CTT Leu	TCA Ser	TTT Phe	TTT Phe	TCA Ser 15	GCA Ala	48
AG7 Ser	GCG Ala	CTG Leu	GCG Ala 20	ACG Thr	CCT Pro	GAT Asp	TGT Cys	GTA Val 25	ACT Thr	GGA Gly	AAG Lys	GTG Val	GAG Glu 30	TAT Tyr	ACA Thr	96
AAA Lys	TAT	AAT Asn 35	GAT Asp	GAC Asp	GAT Asp	ACC Thr	TTT Phe 40	ACA Thr	GTT Val	AAA Lys	GTG Val	GGT Gly 45	GAT Asp	AAA Lys	GAA Glu	144
TTA Leu	TTT Phe 50	ACC Thr	AAC Asn	AGA Arg	TGG Trp	AAT Asn 55	CTT Leu	CAG Gln	TCT Ser	CTT Leu	CTT Leu 60	CTC Leu	AGT Ser	GCG Ala	CAA Gln	192
ATT Ile 65	ACG Thr	GGG Gly	ATG Met	ACT Thr	GTA Val 70	ACC Thr	ATT Ile	AAA Lys	ACT Thr	AAT Asn 75	GCC Ala	TGT Cys	CAT His	AAT Asn	GGA Gl>' 80	240
GGG Gly	GGA Gly	TTC Phe	AGC Ser	GAA Glu	GTT Val	ATT Ile	TTT Phe	CGT Arg						•		267

(2) INFORMATION FOR SEQ ID NO:4:

85

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 89 amino acids
 - (E) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (11) MOLECULE TYPE: protein
- (x1) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala 10

Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr

Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu

Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln

Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly ВО

Gly Gly Phe Ser Glu Val Ile Phe Arg 85

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 954 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

200010 -MIC. CA200+34+ 1 -

(A) NAME/KEY: CDS
(B) LOCATION: 1..954

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATO Met	,-	F TG:	T ATA	A TTX	Pile	AAA Lys	TGG	GTA Val	A CTO	а су	C CT s Le	G TT u Le	TA CI su Le	u G	ST TT Ly Ph	î. ie	48
TCT Ser	TCG Ser	GTZ Val	TCC Ser 20	17.1	TCC Ser	CGG Arg	GAG Glu	TTT Phe 25	Thi	S AT.	A GA e As	C TI P Ph	e Se	G AC r Th	C CA r Gl	A n	96
CAA Gln	AGT Ser	TAI Tyr 35	Val	TCI Ser	TCG	TTA Leu	AAT Asn 40	AGT Ser	ATA Ile	A CGG	G AC.	A GA F Gl 4	u Il	A TC e Se	G AC r Th	C r	144
CCT Pro	CTT Leu 50	G i u	CAT His	ATA Ile	TCT Ser	CAG Gln 55	GGG Gly	ACC Thr	ACA Thr	TCC Ser	GT(Val	Se.	T GT r Va.	T AT l Il	T AA e Asi	C n	192
65	****	nis	Gly	ser	70	TTT Phe	Ala	Val	Asp	75	Arç	Gl;	. Le	ı Ası	9 Val 80	1	240
•			••••	85	nap	CAT His	Dea	Arg	90	116	. 116	GIU	ı Glr	ASI 99	n Asr)	288
	. , .	Val	100	GIÀ	Рпе	GTT Val	Asn	105	Ala	Thr	Asn	Thr	Phe 110	Туг	Arg	1	336
	561	115	File	1111	HIS		ser 120	Val	Pro	Gly	Val	Thr 125	Thr	Val	Ser		384
	130		vah	Set	sei	TAT Tyr 135	inr	Thr	Leu	Gin	Arg 140	Val	Ala	Ala	Leu		432
145	A. 9	361	GIZ	мес	150	ATC :	ser .	Arg	His	Ser 155	Leu	Val	Ser	Ser	Tyr 160		480
	A14	Dea	Met	165	Pne	AGT (Ser (aly .	ASN	Thr 170	Met	Thr	Arg	Asp	Ala 175	Ser		528
··• 9	714	vai	180	Arg	rne	GTC ; Val 1	nr v	Val 185	Thr	Ala	Glu	Ala	Leu 190	Arg	Phe		576
	J	195	GIII .	wid .	9_U		00	iln i	Ala .	Leu	Ser	Glu 205	Thr	Ala	Pro		624
	TAT . Tyr :	ACG Thr	ATG . Met '	ACG :	Pro (GGA G Gly A C15	AC 0	TG (GAČ (Asp)	Leu	ACT Thr 220	CTG Leu	AAC Asn	TGG Trp	Сĵ. ССС		€72

CGA Arg 225	ATC Ile	AGC Ser	AAT Asn	GTG Val	CTT Leu 230	CCG Pro	GAG Glu	TAT Tyr	CGG Arg	GGA Gly 235	GAG Glu	GAT Asp	GGT Gly	GTC Val	AGA Arg 140	720
GTG Val	GGG Gly	AGA Arg	ATA Ile	TCC Ser 245	TTT Phe	AAT Asn	AAT Asn	ATA Ile	TCA Ser 250	GCG Ala	ATA Ile	CTG Leu	GGG Gly	ACT Thr 255	GTG Val	768
GCC Ala	GTT Val	ATA Ile	CTG Leu 260	AAT Asn	TGC Cys	CAT His	CAT His	CAG Gln 265	GGG Gly	GCG Ala	CGT Arg	TCT Ser	GTT Val 270	CGC Arg	GCC Ala	816
GTG Val	AAT Asn	GAA Glu 275	GAG Glu	AGT Ser	CAA Gln	CCA Pro	GAA Glu 280	TGT Cys	CAG Gln	ATA Ile	ACT Thr	GGC Gly 285	GAC Asp	AGG Arg	CCT Pro	864
GTT Val	ATA Ile 190	AAA Lys	ATA Ile	AAC Asn	AAT Asn	ACA Thr 295	TTA Leu	TGG Trp	GAA Glu	AGT Ser	AAT Asn 300	ACA Thr	GCT Ala	GCA Ala	GCG Ala	912
TTT Phe 305	CTG Leu	AAC Asn	AGA Arg	AAG Lys	TCA Ser 310	CAG Gln	TTT Phe	TTA Leu	TAT Tyr	ACA Thr 315	ACG Thr	GGT Gly	AAA Lys			954

(2) INFORMATION FOR SEQ ID NO:6:

180

- :1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 318 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Gly Phe Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Glm Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr
35 40 45 Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg-Gly Leu Asp Val 65 70 75 86 Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser 120 Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu 135 Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr 145 150 155 Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe

Arg	, Glr	7 Ile 19	e Glr	n Arc	g Glu	ı Phe	200	g Gl	n Ala	a Lei	ı Se	r Gl 20	u T!	hr A	la	Pro		
Val	. Tyi	Thi	r Met	The	Pro	Gly 215	Asp	o Va	l Asp	Let	1 Th 22	r Le C	u As	sn T	rp	Gly		
Arg 225	Il€	e Ser	Asn	Val	Let 230	Pro	Glu	ı Tyr	r Arg	Gly 235	Gl	u As	p Gl	y v	al	Arg 240		
Val	Gly	⁄ Arg	, Ile	Ser 245	Phe	Asn	Asn	ılle	Ser 250	Ala	Il	e Le	u Gl		hr 55	Val		
Ala	Val	Ile	Leu 260	Asn	Cys	His	His	Glr 265	Gly	Ala	Arg	g Se:	r Va 27		rg	Ala		
Val	Asn	Glu 275	Glu	Ser	Gln	Pro	Glu 280	Cys	Gln	Ile	Thi	r Gly 289		p Aı	g	Pro		
Val	Ile 290	Lys	Ile	Asn	Asn	Thr 295	Leu	Trp	Glu	Ser	Asr 300	n Thi	- Al	a Al	a	Ala		
Phe 305	Leu	Asn	Arg	Lys	Ser 310	Gln	Phe	Leu	Tyr	Thr 315	Thr	Gly	Ly:	s				
(2)	INF	orma	TION	FOR	SEQ	ID N	JO : 7	:										
	(ix)	() () () () () ()	A) LECUL ATURE A) NA B) LO DUENO	PE: TRANI POLO E TY : ME/K	DEDNI DEDNI DEY: TPE: TEY:	leic ESS: line DNA CDS	acid douk ar (ger	d ole nomi	c)	o: 7 :								
ATG Met 1	AAG Lys	AAG Lys	ATG Met	TTT Phe 5	ATG Met	GCG Ala	GTT Val	TTA Leu	TTT Phe 10	GCA Ala	TTA Leu	GCT Ala	TCT Ser	GTT Val	L A	AT ISD	4	
GCA Ala	ATG Met	GCG Ala	GCG Ala 20	GÁT Asp	TGT Cys	GCT : Ala :	AAA Lys	GGT Gly 25	AAA Lys	ATT	GAG Glu	TTT Phe	TCC Ser 30	AAC Lys	5 T	AT 'y'r	9	•
AAT Asn	GAG Glu	GAT Asp 35	GAC . Asp	ACA Thr	TTT Phe	ACA (Thr \	GTG Val 40	AAG Lys	GTT (GAC (Asp (GGG Gly	AAA Lys 45	GAA Glu	TAC Tyr	T	GG rp	14	4
ACC Thr	AGT Ser 50	CGC Arg	TGG /	AAT Asn :	CTG Leu	CAA (Gln I 55	CCG '	TTA Leu	CTG (Leu (CAA A Sln S	GT Ser 60	GCT Ala	CAG Gln	TTG Leu	A)	CA hr	15:	-
GGA . Gly 1 65	ATG Met	ACT Thr	GTC /	ACA I	ATC : Ile : 70	AAA 1 Lys 9	rcc : Ser :	AGT . Ser	ACC T	Cys C	SAA Slu	TCA Ser	GGC GGC	TCC Ser	G.	3A 1 <u>y</u> 8 0	240	-
he i	GCT Ala	GAA Glu	GTG (Val (CAG : Sln 1	TTT : Phe :	AAT A Asn A	AT (GAC Asp		•							267	;

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 89 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEO ID NO:8:

Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn

Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr

Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp

Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr 50

Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly

Phe Ala Glu Val Gln Phe Asn Asn Asp 85

- (2) INFORMATION FOR SEQ ID NO:9:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1241 base pairs

 - (B) TYPE: nucleic acid (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGAAAATAA TTATTTTTAG AGTGCTAACT TTTTTCTTTG TTATCTTTTC AGTTAATGTG 60 GTGGCGAAGG AATTTACCTT AGACTTCTCG ACTGCAAAGA CGTATGTAGA TTCGCTGAAT 120 GTCATTCGCT CTGCAATAGG TACTCCATTA CAGACTATTT CATCAGGAGG TACGTCTTTA 180 CTGATGATTG ATAGTGGCTC AGGGGATAAT TTGTTTGCAG TTGATGTCAG AGGGATAGAT 240 GCAGAGGAAG GGCGGTTTAA TAATCTACGG CTTATTGTTG AACGAAATAA TTTATATGTG 300 ACAGGATTTG TTAACAGGAC AAATAATGTT TTTTATCGCT TTGCTGATTT TTCACATGTT 360 ACCTTTCCAG GTACAACAGC GGTTACATTG TCTGGTGACA GTAGCTATAC CACGTTACAG 420 CGTGTTGCAG GGATCAGTCG TACGGGGATG CAGATAAATC GCCATTCGTT GACTACTTCT 480 TATCTGGATT TAATGTCGCA TAGTGGAACC TCACTGACGC AGTCTGTGGC AAGAGCGATG 540 TTACGGTTTG TTACTGTGAC AGCTGAAGCT TTACGTTTTC GGCAAATACA GAGGGGATTT 600 CGTACAACAC TGGATGATCT CAGTGGGCGT TCTTATGTAA TGACTGCTGA AGATGTTGAT 660 CTTACATTGA ACTGGGGAAG GTTGAGTAGC GTCCTGCCTG ACTATCATGG ACAAGACTCT 720 GTTCGTGTAG GAAGAATTTC TTTTGGAAGC ATTAATGCAA TTCTGGGAAG CGTGGCATTA 780 ATACTGAATT GTCATCATCA TGCATCGCGA GTTGCCAGAA TGGCATCTGA TGAGTTTCCT 64 C TCTATGTGTC CGGCAGATGG AAGAGTCCGT GGGATTACGC ACAATAAAAT ATTGTGGGAT 900

TCATCCACTC	TGGGGGCAAT	TCTGATGCGC	AGAACTATTA	GCAGTTGAAC	AGGGGGTAAA	960
TAAAGGAGTT	AAGCATGAAA	AAAACATTAT	TAATAGCTGC	ATCGCTTTCA	TTTTTTTCAG	1020
CAAGTGCGCT	GGCGACGCCT	GATTGTGTAA	CTGGAAAGGT	GGAGTATACA	AAATATAATG	1080
ATGACGATAC	CTTTACAGTT	AAAGTGGGTG	ATAAAGAATT	ATTTACCAAC	AGATGGAATC	1140
TTCAGTCTCT	TCTTCTCAGT	GCGCAAATTA	CGGGGATGAC	TGTAACCATT	AAAACTAATG	1200
CCTGTCATAA	TGGAGGGGGA	TTCAGCGAAG	TTATTTTTCG	Τ		1241

- (2) INFORMATION FOR SEQ ID NO:10:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1235 base pairs

 - (B) TYPE: nucleic acid (C) STRANDEDNESS: double (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATGAAGTGTA	. TATTATTE	ATGGGTACT	TGCCTGTTAC	TGGGTTTTT	TTCGGTATCC	60
TATTCCCGGG	AGTTTACGAT	AGACTTTTCC	ACCCAACAA	GTTATGTCTC	TTCGTTAAAT	120
AGTATACGGA	CAGAGATATO	GACCCCTCTT	GAACATATAT	CTCAGGGGAC	CACATCGGTG	180
TCTGTTATTA	ACCACACOCA	CGGCAGTTAT	TTTGCTGTGG	ATATACGAGG	GCTTGATGTC	240
TATCAGGCGC	GTTTTGACCA	. TCTTCGTCTG	ATTATTGAGO	AAAATAATTT	ATATGTGGCA	300
GGGTTCGTTA	ATACGGCAAC	AAATACTTTC	TACCGTTTTT	CAGATTTTAC	ACATATATCA	360
GTGCCCGGTG	TGACAACGGT	TTCCATGACA	ACGGACAGCA	GTTATACCAC	TCTGCAACGT	420
GTCGCAGCGC	TGGAACGTTC	CGGAATGCAA	ATCAGTCGTC	ACTCACTGGT	TTCATCATAT	480
CTGGCGTTAA	TGGAGTTCAG	TGGTAATACA	ATGACCAGAG	ATGCATCCAG	AGCAGTTOTG	54 C
			CGCTTCAGGC			600
			ACGATGACGC			660
			CCGGAGTATC			720
			GCGATACTGG			780
			CGCGCCGTGA			
			AAAATAAAA			840
						900
			CAGTTTTTAT		TAAATAAAGG	960
AGTTAAGCAT	GAAGAAGATG	TTTATGGCGG	TTTTATTTGC	ATTAGCTTCT	GTTAATGCAA	1020
TGGCGGCGGA	TTGTGCTAAA	GGTAAAATTG	AGTTTTCCAA	GTATAATGAG	GATGACACAT	1080
TTACAGTGAA	GGTTGACGGG	AAAGAATACT	GGACCAGTCG	CTGGAATCTG	CAACCGTTAC	1140
TGCAAAGTGC	TCAGTTGACA	GGAATGACTG	TCACAATCAA	ATCCAGTACC	TGTGAATCAG	1200
GCTCCGGATT	TGCTGAAGTG	CAGTTTAATA	ATGAC			1235

- (2) INFORMATION FOR SEQ ID NO:11:
 - (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 6 amino acids

(B) TYPE: amino acid(C) STRANDEDNESS: unknown(D) TOPOLOGY: linear		
(ii) MOLECULE TYPE: peptide		
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:		
Leu Glu His His His His His 1		
(2) INFORMATION FOR SEQ ID NO:12:		
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 29 base pairs (E) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear		
(ii) MOLECULE TYPE: DNA (genomic)		
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:		
GCCATATGAA AATAATTATT TTTAGAGTG		2.9
(2) INFORMATION FOR SEQ ID NO:13:		
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 29 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear		
(ii) MOLECULE TYPE: DNA (genomic)		
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:		
GGCTCGAGAC TGCTAATAGT TCTGCGCAT		29
(2) INFORMATION FOR SEQ ID NO:14:		
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 28 base pairs (B: TYPE: nucleic acid :C: STRANDEDNESS: single :D: TOPOLOGY: linear		
(ii) MCLECULE TYPE: DNA (genomic)		
(x1) SEQUENCE DESCRIPTION: SEQ ID NO:14:		
GCCATATGAA AAAAACATTA TTAATAGC .		28
(2) INFORMATION FOR SEQ ID NO:15:		
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 29 base pairs (E) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear		
(ii) MCLECULE TYPE: DNA (genomic)		
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:		
GCTCGAGAC GAAAATAAC TTCGCTGAA		29
2) INFORMATION FOR SEQ ID NO:16:	4	
(1) SEQUENCE CHARACTERISTICS:		

(A) LENGTH: 29 base pairs(B) TYPE: nucleic acid(C) STRANDEDNESS: single(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:	
GCCATATGAA GTGTATATTA TTTAAATGG	2
(2) INFORMATION FOR SEQ ID NO:17:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 30 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:	
GGCTCGAGTT TACCCGTTGT ATATAAAAC	3 0
(2) INFORMATION FOR SEQ ID NO:18:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 26 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:	
CGCATATGAA GAAGATGTTT ATGGCG	26
(2) INFORMATION FOR SEQ ID NO:19:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 19 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(11) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:	
GGCTCGAGGT CATTATTAAA CTGCACTTC	29
(2) INFORMATION FOR SEQ ID NO:20:	
(1) SEQUENCE CHARACTERISTICS: (A) LENGTH: 969 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(1X) FEATURE: (A) NAME/KEY: CDS (B) LOCATION: 1969	
(X1) SEQUENCE DESCRIPTION: SEQ ID NO:20:	
ATG AAA ATA ATT ATT TTT AGA GTG CTA ACT TTT TTC TTT GTT ATC TTT	48

Me	t Ly 1	s Il	e Il	e Ile	e Pho	e Arg	y Val	l Le	u Th:	r Phe	≘ Ph	e Ph	e Va		e Phe	e
TC. Se	A GT r Va	T AA l As	T GTO n Val	ı va.	G GCC l Ala	G AAC a Lys	G GAJ G Glu	A TT: 1 Phe 2!	e Thi	TTA Lev	A GA 1 As	C TT p Ph	C TC e Se 3	r Th	T GCA	A 96
AA(Ly:	G AC	G TATE	r va.	A GA1 l Asp	TCC Ser	CTC Lev	AAT Asn 40	ı Val	T ATT	CGC Arg	TC' Se:	T GC. r Ala	a Il	A GG e Gl	T ACT y Thr	144
CC; Pro	A TTZ D Let 50	a Gi:	ACT Thi	r ATT	TCA Ser	TCA Ser 55	Gly	GGT Gly	ACG Thr	TCT Ser	TT: Lev	ı Le	G ATG	S AT	T GAT e Asp	192
AGT Ser 65	. 617	TC) Sei	GGG Gly	GAT / Asp	AAT ASD 70	Leu	TTT Phe	GCA Ala	GTT Val	GAT Asp 75	Va]	AGA L Arg	GGG GGI	AT.	A GAT e Asp 80	
GCA Ala	GAC Glu	GAA Glu	GGG Gly	CGG Arg 85	Phe	AAT Asn	AAT Asn	CTA Leu	CGG Arg 90	Leu	ATT Ile	GTT Val	GAA Glu	CG Arg	A AAT g Asn	288
AAT Asn	TTA Leu	TAT Tyr	GTG Val 100	Thr	GGA Gly	TTT	GTT Val	AAC Asn 105	Arg	ACA Thr	AAT Asn	AAT Asn	GTT Val	Phe	TAT Tyr	33€
CGC Arg	TTT Phe	GCT Ala 115	Asp	TTT Phe	TCA Ser	CAT His	GTT Val 120	ACC Thr	TTT Phe	CCA Pro	GGT Gly	ACA Thr 125	ACA Thr	GCC Ala	GTT Val	384
ACA Thr	TTG Leu 130	Ser	GGT Gly	GAC Asp	AGT Ser	AGC Ser 135	TAT Tyr	ACC Thr	ACG Thr	TTA Leu	CAG Gln 140	Arg	GTT Val	GCA Ala	GGG Gly	432
ATC Ile 145	AGT Ser	CGT Arg	ACG Thr	GGG Gly	ATG Met 150	CAG Gln	ATA Ile	AAT Asn	CGC Arg	CAT His 155	TCG Ser	TTG Leu	ACT Thr	ACT Thr	TCT Ser 160	480
TAT Tyr	CTG Leu	GAT Asp	TTA Leu	ATG Met 165	TCG Ser	CAT His	AGT Ser	GGA Gly	ACC Thr 170	TCA Ser	CTG Leu	ACG Thr	CAG Gln	TCT Ser 175	STG Val	528
GCA Ala	AGA Arg	GCG Ala	ATG Met 180	TTA Leu	CGG Arg	TTT Phe	GTT Val	ACT Thr 185	GTG Val	ACA Thr	GCT Ala	GAA Glu	GCT Ala 190	TTA Leu	CGT Arg	57€
TTT	CGG Arg	CAA Gln 195	ATA Ile	CAG Gln	AGG Arg	GGA Gly	TTT Phe 200	CGT Arg	ACA Thr	ACA Thr	CTG Leu	GAT Asp 205	GAT Asp	CTC Leu	AGT Ser	624
GGG Gly	CGT Arg 210	TCT Ser	TAT Tyr	GTA Val	ATG Met	ACT Thr 215	GCT Ala	GAA Glu	GAT Asp	Val.	GAT Asp 220	CTT Leu	ACA Thr	TTG Leu	AAC Asn	672
TGG Trp 225	GGA Gly	AGG Arg	TTG Leu	Ser	AGC Ser 230	GTC Val	CTG Leu	CCT Pro	GAC Asp	TAT Tyr 235	CAT His	GGA Gly	CAA Gln	GAC Asp	TCT Ser 240	720
GTT Val	CGT Arg	GTA Val	GGA Gly	AGA Arg 245	ATT Ile	TCT Ser	TTT Phe	Gly	AGC Ser 250	ATT I	AAT Asn	GCA Ala	Ile	CTG Leu 255	GGA Gly	768
AGC Ser	GTG Val	GCA Ala	TTA Leu 260	ATA Ile	CTG Leu	AAT ' Asn (Cys I	CAT His 265	CAT (CAT (GCA Ala	Ser	CGA Arg 270	GTT Val	GCC Ala	81€
AGA Arg	ATG Met	GCA Ala 275	TCT Ser	GAT (Asp (GAG ' Glu	Phe 1	CCT 1 Pro 1 280	ICT . Ser .	ATG '	TGT (Cys I	Pro	GCA Ala 285	GAT Asp	GGA Gly	AGA Arg	66 ;

GTC Val	CGT Arg 290	GGG Gly	ATT	ACG Thr	CAC H1s	AAT Asn 295	Lys	ATA Ile	TTG Leu	TGG Trp	GAT Asp 300	TCA Ser	TCC Ser	ACT Thr	CTG Leu	911
GGG Gly 305	GCA Ala	ATT Ile	CTG Leu	ATG Met	CGC Arg 310	AGA Arg	ACT Thr	ATT Ile	AGC Ser	AGT Ser 315	CTC Leu	GAG Glu	CAC His	CAC His	CAC His 320	960
	CAC His	CAC His														969

- (2) INFORMATION FOR SEQ ID NO:21:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 323 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21: Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser 155 Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg

185 Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn 210 215 Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser

. Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly 250

SECURIO SINIO GRADINANA I .

Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg 280 Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu 295 Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser Leu Glu His His His 305 310 315 His His His (2) INFORMATION FOR SEQ ID NO:22: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 294 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (ix) FEATURE: (A) NAME/KEY: CDS (B) LOCATION: 1..294 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22: ATG AAA AAA ACA TTA TTA ATA GCT GCA TCG CTT TCA TTT TTT TCA GCA 48 Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala าก AGT GCG CTG GCG ACG CCT GAT TGT GTA ACT GGA AAG GTG GAG TAT ACA Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr 96 AAA TAT AAT GAT GAC GAT ACC TTT ACA GTT AAA GTG GGT GAT AAA GAA 144 Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu 40 TTA TTT ACC AAC AGA TGG AAT CTT CAG TCT CTT CTC AGT GCG CAA 192 Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln 55 ATT ACG GGG ATG ACT GTA ACC ATT AAA ACT AAT GCC TGT CAT AAT GGA lie Thr Gly Met Thr Val Thr lie Lys Thr Asn Ala Cys His Asn Gly 240 GGG GGA TTC AGC GAA GTT ATT TTT CGT CTC GAG CAC CAC CAC CAC Gly Gly Phe Ser Glu Val Ile Phe Arg Leu Glu His His His His 288 25 90 CAC TG 294 His

- (2) INFORMATION FOR SEQ ID NO:23:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 97 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein
 - (x1) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala

10 Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Leu Ser Ala Gln 55 Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly Gly Gly Phe Ser Glu Val Ile Phe Arg Leu Glu His His His His His 90 His (2) INFORMATION FOR SEQ ID NO:24: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 981 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (11) MOLECULE TYPE: DNA (genomic) (ix) FEATURE: (A) NAME/KEY: CDS (B) LOCATION: 1..981 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24: ATG AAG TGT ATA TTA TTT AAA TGG GTA CTG TGC CTG TTA CTG GGT TTT Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Gly Phe ÷8 TCT TCG GTA TCC TAT TCC CGG GAG TTT ACG ATA GAC TTT TCG ACC CAA Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln 96 CAA AST TAT GTC TCT TCG TTA AAT AGT ATA CGG ACA GAG ATA TCG ACC Gin Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr CCT CTT GAA CAT ATA TCT CAG GGG ACC ACA TCG GTG TCT GTT ATT AAC 192 Pro Leu Glu His Ile Ser Glm Gly Thr Thr Ser Val Ser Val Ile Asm 5.5 CAC ACC CAC GGC AGT TAT TTT GCT GTG GAT ATA CGA GGG CTT GAT GTC His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val 240 TAT CAG GCG CGT TTT GAC CAT CTT CGT CTG ATT ATT GAG CAA AAT AAT Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn 288 TTA TAT GTG GCA GGG TTC GTT AAT ACG GCA ACA AAT ACT TTC TAC CGT Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg 100 TIT TOA GAT TIT ACA CAT ATA TOA GTG COO GGT GTG ACA ACG GTT TOO 384 Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser 120 ATG ACA ACG GAC AGC AGT TAT ACC ACT CTG CAA CGT GTC GCA GCG CTG Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu 432 140

SUCCES THE GESTIONS . . .

GAJ Glu 145	ı Arg	TCC Ser	GGA Gly	ATG Met	Gln 150	Ile	AGT Ser	CGT Arg	CAC His	TCA Ser 155	Leu	GTT Val	TC; Ser	TC/ Sei	TAT Tyr 160	480
CTC	GCG Ala	TTA Leu	ATG Met	GAG Glu 165	Phe	AGT Ser	GGT Gly	AAT Asn	ACA Thr 170	Met	ACC Thr	AGA Arg	GAT Asp	GCA Ala 175	TCC Ser	528
AGA Arg	GCA Ala	GTT Val	CTG Leu 180	CGT Arg	TTT Phe	GTC Val	ACT Thr	GTC Val 185	ACA Thr	GCA Ala	GAA Glu	GCC Ala	TTA Leu 190	Arg	TTC Phe	57€
AGG Arg	CAG Gln	ATA Ile 195	CAG Gln	AGA Arg	GAA Glu	TTT Phe	CGT Arg 200	CAG Gln	GCA Ala	CTG Leu	TCT Ser	GAA Glu 205	ACT Thr	GCT Ala	CCT	624
GTG Val	TAT Tyr 210	ACG Thr	ATG Met	ACG Thr	CCG Pro	GGA Gly 215	GAC Asp	GTG Val	GAC Asp	CTC Leu	ACT Thr 220	CTG Leu	AAC Asn	TGG Trp	GGG Gly	672
CGA Arg 225	ATC Ile	AGC Ser	AAT Asn	GTG Val	CTT Leu 230	CCG Pro	GAG Glu	TAT Tyr	CGG Arg	GGA Gly 235	GAG Glu	GAT Asp	GGT Gly	GTC Val	AGA Arg 240	720
GTG Val	GGG Gly	AGA Arg	ATA Ile	TCC Ser 245	TTT Phe	AAT Asn	AAT Asn	ATA Ile	TCA Ser 250	GCG Ala	ATA Ile	CTG Leu	GGG Gly	ACT Thr 255	GTG Val	768
GCC Ala	GTT Val	ATA Ile	CTG Leu 260	AAT Asn	TGC Cys	CAT His	CAT His	CAG Gln 265	GGG Gly	GCG Ala	CGT Arg	TCT Ser	GTT Val 270	CGC Arg	GCC Ala	816
GTG Val	AAT Asn	GAA Glú 275	GAG Glu	AGT Ser	CAA Gln	CCA Pro	GAA Glu 280	TGT Cys	CAG Gln	ATA Ile	ACT Thr	GGC Gly 285	GAC Asp	AGG Arg	CCT Pro	864
GTT Val	ATA Ile 290	AAA Lys	ATA Ile	AAC Asn	AAT Asn	ACA Thr 295	TTA Leu	TGG Trp	GAA Glu	AGT Ser	AAT Asn 300	ACA Thr	GCT Ala	GCA Ala	GCG Ala	912
TTT Phe	CTG Leu	AAC Asn	AGA Arg	AAG Lys	TCA Ser 310	CAG Gln	TTT Phe	TTA Leu	Tyr	ACA Thr 315	ACG Thr	GGT Gly	AAA Lys	CTC Leu	GAG Glu 320	960
CAC His	CAC His	CAC His	CAC His	CAC His 325	CAC Hıs	TG										981

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 326 amino acids (B) TYPE: amino acid

 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe

Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gin

Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr 35 .40 45

Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn 55

His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val

Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser 120 Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu 135 Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser 165 170 Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe 180 185 Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro 200 Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly 215 Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala 265 Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro 280 Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala 295

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- (2) INFORMATION FOR SEQ ID NO:26:
 - (2) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 294 base pairs
 - (E) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..294
 - (x1) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATG AAG AAG ATG TIT ATG GCG GTT TIA TITT GCA TIA GCT TOT GTT AAT Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn 1 5 10 15

Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys Leu Glu

GCA Ala	ATG Met	GCG Ala	GCG Ala 20	Asp	TGT Cys	GCT Ala	AAA Lys	GGT Gly 25	lys	ATT Ile	GAG Glu	TTT Phe	TCC Ser 30	Lys	TAT Tyr	96
AAT Asn	GAG Glu	GAT Asp 35	GAC Asp	ACA Thr	TTT Phe	ACA Thr	GTG Val 40	AAG Lys	GTT Val	GAC Asp	GGG Gly	AAA Lys 45	GAA Glu	TAC Tyr	TGG Trp	144
ACC Thr	AGT Ser 50	CGC	TGG Trp	AAT Asn	CTG Leu	CAA Gln 55	CCG Pro	TTA Leu	CTG Leu	CAA Gln	AGT Ser 60	GCT Ala	CAG Gln	TTG Leu	ACA Thr	192
GGA Gly 65	ATG Met	ACT Thr	GTC Val	ACA Thr	ATC Ile 70	AAA Lys	TCC Ser	AGT Ser	ACC Thr	TGT Cys 75	GAA Glu	TCA Ser	GGC Gly	TCC Ser	GGA Gly 60	240
TTT Phe	GCT Ala	GAA Glu	GTG Val	CAG Gln 85	TTT Phe	AAT Asn	AAT Asn	GAC Asp	CTC Leu 90	GAG Glu	CAC His	CAC . His	CAC His	CAC His 95	CAC H1s	288
CAC His	TG															294

- (2) INFORMATION FOR SEQ ID NO:27:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 97 amino acids(B) TYPE: amino acid(D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn

Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr

Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp

Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr

Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly

Phe Ala Glu Val Gln Phe Asn Asn Asp Leu Glu His His His His 85

His

- (2) INFORMATION FOR SEQ ID NO:28:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (E) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 - (ii) MCLECULE TYPE: DNA (genomic)
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGGAATTCAA GGAATTTACC TTAGACTTCT CG

(2) INFORMATION FOR SEQ ID NO:29:

 (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 28 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear 	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:	
GGCTCGAGTC AACTGCTAAT AGTTCTGC	28
(2) INFORMATION FOR SEQ ID NO:30:	
 (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 32 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear 	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:	
CGGAATTCCG GGAGTTTACG ATAGACTTTT CG	32
(2) INFORMATION FOR SEQ ID NO:31:	•
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 29 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:	
GGCTCGAGTT ATTTACCCGT TGTATATAA	29
(2) INFORMATION FOR SEQ ID NO:32:	
(1) SEQUENCE CHARACTERISTICS: (A) LENGTH: 2127 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(ix) FEATURE: (A) NAME/KEY: CDS (B) LOCATION: 12127	
(%1) SEQUENCE DESCRIPTION: SEQ ID NO:31:	
ATG AAA ATA AAA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala 1 5 10	TTA ACG 46 Leu Thr 15
ACG ATG ATG TTT TCC GCC TCG GCT CTC GCC AAA ATC GAA GAA Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu 20 25 30	GGT AAA 96 Gly Lys
TTG GTA ATC TGG ATT AAC GGC GAT AAA GGC TAT AAC GGT CTC Leu Val Ile Trp lle Asn Gly Asp Lys Gly Tyr Asn Gly Leu 35 40 45	GCT GAA 144 Ala Glu
STC GGT AAG AAA TTC GAG AAA GAT ACC GGA ATT AAA GTC ACC. Val Gly Lys Lys Phe Gly Lys Asp Thy Gly Tie Lys Yal Thy	GTT GAG 192

	5 ()				5.5	5				6)				
CAT His	Pro	G GAT	Lys	A CTO	G GAA u Glu 70	ı Glu	AAA Lys	TTO Phe	C CCA	CAC Glr 75	ı Val	r GCG	G GC	A AC a Th	T GGC F Gly	240
ASP	, Già	Pro	Asp	85	: Ile	Phe	Trp) Ala	His 90	Asp	Arç	; Phe	e Gly	/ G1:	_	288
GCT Ala	CAA Gln	TCT Ser	GGC Gly 100	Leu	TTG Leu	GCT Ala	GAA Glu	Ile 105	Thr	CCG Pro	GAC Asp	Lys	A GCC s Ala 110	Phe	CAG Gln	336
Asp	Lys	Leu 115	Тут	Pro	Phe	Thr	Trp 120	Asp	Ala	Val	Arg	Tyr 125	Asr	Gly	AAG Lys	384
CTG Leu	ATT Ile 130	GCT Ala	TAC	CCG Pro	ATC Ile	GCT Ala 135	GTT Val	GAA Glu	GCG Ala	TTA Leu	TCG Ser 140	CTG Leu	ATT	TAT	AAC Asn	432
AAA Lys 145	GAT Asp	CTG Leu	CTG Leu	CCG Pro	AAC Asn 150	CCG Pro	CCA Pro	AAA Lys	ACC Thr	TGG Trp 155	GAA Glu	GAG Glu	ATC Ile	CCG	GCG Ala 160	480
CTG Leu	GAT Asp	AAA Lys	GAA Glu	CTG Leu 165	AAA Lys	GCG Ala	AAA Lys	GGT Gly	AAG Lys 170	AGC Ser	GCG Ala	CTG Leu	ATG Met	TTC Phe 175	AAC Asn	528
Leu	Gln	Glu	Pro 180	Tyr	Phe	Thr	Trp	Pro 185	Leu	Ile	Ala	Ala	Asp 190	Gly	GGT Gly	576
Tyr	Ala	Phe 195	Lys	Tyr	GAA Glu	Asn	Gly 200	Lys	Tyr	Asp	Ile	Lys 205	Asp	Val	Gly	624
vaI	210	Asn	Ala	Gly	GCG Ala	Lys 215	Ala	Gly	Leu	Thr	Phe 220	Leu	Val	Asp	Leu	672
11e 225	Lys	Asn	Lys	His	ATG Met 230	Asn	Ala	Asp	Thr	Asp 235	Tyr	Ser	Ile	Ala	Glu 240	720
Ala	Ala	Phe	Asn	Lys 245	GGC	Glu	Thr	Ala	Met 250	Thr	Ile	Asn	Gly	Pro 255	Trp	76 ė
GCA Ala	Trp	Ser	Asn 260	Ile	Asp	Thr	Ser	Lys 2 6 5	Val	Asn	Tyr	Gly	Val 270	Thr	Val	816
CTG Leu	Pro	Thr 275	Phe	Lys	Gly	Gln	Pro 280	Ser	Lys	Pro	Phe	Val 285	Gly	Val	Leu	86;
AGC Ser	GCA Ala 290	GGT Gly	ATT Ile	AAC Asn	Ala .	GCC . Ala . 295	AGT Ser	CCG Pro	AAC . Asn .	Lys ·	GAG Glu 300	CTG Leu	GCG Ala	AAA Lys	GAG Glu	912

TT Ph	e re	C GA u Gl	A AA u As:	C TA'	T CTO r Leu 310	ı Lei	AC: Thi	T GA	T GA p Gl	A GG u Gl 31	y Le	G G;	iu Al	CG G La V	TT	AAT Asn 320		960
AA: Ly:	A GA S Asj	C AAJ p Lys	A CCO	G CT(D Lev 32:	1 GT?	C GCC Ala	GTA Val	A GC0 . Ala	G CTG a Let 330	u Ly:	G TC s Se	T TA	c GA r Gl	u G	AA lu 35	GAG Glu		1008
TT(Lev	G GCC	a Lys	A GAT S ASI 340	o Pro	CGT Arg	ATT	GCC Ala	GC0 Ala 345	a Thi	T ATO	G GA	A AA u As	C GC n Al 35	a G	AG ln	AAA Lys		1056
GG7 Gly	GAA Glu	A ATO	e Met	CCG Pro	AAC Asn	ATC Ile	CCG Pro 360	Glr	ATO Met	TCC Ser	G GC	T TT a Ph 36	e Tr	G TA	AT 'r	GCC Ala		1104
GT(Val	CGT Arg 370	ACI Thr	GCG Ala	GTG Val	ATC Ile	AAC Asn 375	GCC Ala	GCC Ala	AGC Ser	GGT Gly	CG' 'Arg	g Gl:	G AC	T GI r Va	C 1	GAT Asp		1152
GAA Glu 385	ATS	CTC Leu	AAA Lys	GAC Asp	GCG Ala 390	CAG Gln	ACT Thr	TCG Ser	AGC Ser	TCG Ser 395	Asr	C AA	C AA n Ası	C AA n As	n i	AAT Asn 400		1200
AAC Asn	AAT Asn	AAC Asn	AAC Asn	AAC Asn 405	CTC Leu	GGG Gly	ATC Ile	GAG Glu	GGA Gly 410	Arg	ATT Ile	TC: Sei	A GAJ	A TT Ph 41	e i	AAG Lys		1246
GAA Glu	TTT	ACC Thr	TTA Leu 420	Asp	TTC Phe	TCG Ser	ACT Thr	GCA Ala 425	AAG Lys	ACG Thr	TAT Tyr	GT; Val	A GAT Asp 430	Se	G (CTG Leu		1296
AAT Asn	GTC Val	ATT Ile 435	CGC	TCT Ser	GCA Ala	ATA Ile	GGT Gly 440	ACT Thr	CCA Pro	TTA Leu	CAG Gln	ACT Thr 445	Ile	TC: Se:	A I	CCA Ser		1344
GGA Gly	GGT Gly 450	ACG Thr	TCT Ser	TTA Leu	CTG Leu	ATG Met 455	ATT Ile	GAT Asp	AGT Ser	GGC Gly	TCA Ser 460	GGG Gly	GAT Asp	AAT Asr	r r	TG eu		1392
TTT Phe 465	GCA Ala	GTT Val	GAT Asp	GTC Val	AGA Arg 470	GGG Gly	ATA Ile	GAT Asp	GCA Ala	GAG Glu 475	GAA Glu	GGG Gly	CGG Arg	TTI Phe	A	AT sn 80		1440
ASII	Leu	CGG Arg	Leu	11e 485	Val	Glu	Arg	Asn	Asn 490	Leu	Tyr	Val	Thr	Gly 495	P.	he		1488
GTT Val	AAC Asn	AGG Arg	ACA Thr 500	AAT Asn	AAT Asn	GTT Val	TTT Phe	TAT Tyr 505	CGC Arg	TTT Phe	GCT Ala	GAT Asp	TTT Phe 510	TCA Ser	C: H:	AT is	:	1536
GTT Val	ACC Thr	TTT Phe 515	CCA Pro	GGT Gly	ACA Thr	Thr .	GCG Ala 520	GTT Val	ACA Thr	TTG Leu	TCT Ser	GGT Gly 525	GAC Asp	AGT Ser	A(Se	GC er	נ	.584
TAT	ACC Thr 530	ACG Thr	TTA Leu	CAG Gln	Arg	GTT (Val , 535	GCA Ala	GGG Gly	ATC Ile	Ser	CGT Arg 540	ACG Thr	GGG Gly	ATG Met	C# G]	AG Ln	נ	632
ATA Ile 545	AAT Asn	CGC Arg	CAT His	Ser	TTG . Leu : 550	ACT Z	ACT :	TCT Ser	Tyr	CTG Leu 555	GAT Asp	TTA Leu	ATG Met	TCG Ser	CA H1 56	. s	1	680
AGT Ser	GGA Gly	ACC Thr	TCA Ser	CTG Leu 565	ACG (Thr (CAG : Sln :	TCT (Ser '	Val .	GCA . Ala . 570	AGA (Arg .	GCG Ala	ATG Met	TTA Leu	CGG Arg 575	TT Ph	T ie	1	728 .
GTT Val	ACT Thr	GTG Val	ACA Thr	GCT : Ala :	GAA (Glu /	GCT :	TTA (Leu)	GT Arg	TTT (Phe)	CGG (Arg (CAA Gln	ATA Ile	CAG Gln	AGG Arg	GG G1	A Y	1	77€

PENNOTE AND CONGRESS ...

580 585 590 TTT CGT ACA ACA CTG GAT GAT CTC AGT GGG CGT TCT TAT GTA ATG ACT Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr 1824 595 600 GCT GAA GAT GTT GAT CTT ACA TTG AAC TGG GGA AGG TTG AGT AGC GTC Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val 1872 620 CTG CCT GAC TAT CAT GGA CAA GAC TCT GTT CGT GTA GGA AGA ATT TCT Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser 1920 630 TTT GGA AGC ATT AAT GCA ATT CTG GGA AGC GTG GCA TTA ATA CTG AAT 1968 Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn 650 TGT CAT CAT GCA TCG CGA GTT GCC AGA ATG GCA TCT GAT GAG TTT 2016 Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe 660 665 CCT TCT ATG TGT CCG GCA GAT GGA AGA GTC CGT GGG ATT ACG CAC AAT 2064 Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr His Asn 675 680 AAA ATA TTG TGG GAT TCA TCC ACT CTG GGG GCA ATT CTG ATG CGC AGA 2112 Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met Arg Arg 695 700 ACT ATT AGC AGT TG 2127 Thr Ile Ser Ser

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 708 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr

Thi Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys
20 25 30

Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu 35 40 . 45

Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu 50 60

His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly
65 70 75 80

Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr

Ala Glr. Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln
100 105 110

Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys

Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn

	13	0				139	5				14	O			
Ly 14	s As 5	p Le	u Le	u Pr	0 Asn 150	Pro	o Pr	o Lys	s Th	r Tr 15	p Gl 5	u Gl	u I	le P	ro Ala 160
Le	u As	p Ly	s Gl	u Le: 16	u Lys 5	Ala	a Ly:	s Gly	, Ly	s Se O	r Al	a Le	u Me		he Asn 75
Le	u Gl:	n Gl	u Pro	с Ту: 0	r Phe	Thr	Tr	P Pro	Le	u Il	e Al	a Al	a As		ly Gly
Ту	r Ala	a Ph 19	e Ly: 5	s Tyr	r Glu	Asn	Gly 200	/ Lys	ту:	r As	p Il	e Ly 20		p Vē	al Gly
Va	l Ası 210	p As:	n Ala	a Gly	/ Ala	Lys 215	Ala	Gly	· Le	u Th:	Phe 220		u Va	l As	p Leu
116 22	e Lys	s Ası	n Lys	5 His	Met 230	Asn	Ala	Asp	Thi	r Asp 235		s Se	r Il	e Al	a Glu 240
Ala	a Ala	a Phe	e Asr	1 Lys 245	Gly	Glu	Thr	Ala	Met 250	Th:	Ile	Ası	n Gl	y Pr 25	o Trp 5
Ala	a Trp	Se s	260	lle	Asp	Thr	Ser	Lys 265	Val	. Asn	туг	Gly	/ Va.		r Val
Let) Pro	Thi 275	Phe	Lys	Gly	Gln	Pro 280	Ser	Lys	Pro	Phe	Val 285		y Va.	l Leu
Ser	Ala 290	Gly	'Ile	Asn	Ala	Ala 295	Ser	Pro	Asn	Lys	Glu 300	Leu	Ala	a Lys	s Glu
Phe 305	Leu	Glu	Asn	Tyr	Leu 310	Leu	Thr	Asp	Glu	Gly 315	Leu	Glu	Ala	Val	l Asn 320
Lys	Asp	Lys	Pro	Leu 325	Gly	Ala	Val	Ala	Leu 330	Lys	Ser	Tyr	Glu	Glu 335	
Leu	Ala	Lys	Asp 340	Pro	Arg	Ile	Ala	Ala 345	Thr	Met	Glu	Asn	Ala 350		Lys
Gly	Glu	Ile 355	Met	Pro	Asn	Ile	Pro 360	Gln	Met	Ser	Ala	Phe 365	Trp	Tyr	Ala
Val	Arg 370	Thr	Ala	Val	Ile .	As n 375	Ala	Äla	Ser	Glγ	Arg 380	Gln	Thr	Val	Asp
Glu 385	Ala	Leu	Lys	Asp	Ala (Gln	Thr	Ser	Ser	Ser 395	Asn	Asn	Asn	Asn	Asn 400
Asn	Asn	Asn	Asn	Asn 405	Leu (Gly	Ile	Glu	Gly 410	Arg	lle	Ser	Glu	Phe 415	Lys
Glu	Phe	Thr	Leu 420	Asp	Phe :	Ser	Thr	Ala 425	Lys	Thr	Tyr	Val	Asp 430	Ser	Leu
Asn	Val	Ile 435	Arg	Ser	Ala 1	lle	Gly 440	Thr :	Pro	Leu	Gln	Thr 445	Ile	Ser	Ser
Gly	Gly 450	Thr	Ser	Leu	Leu A	1et :	lle	Asp :	Ser	Gly	Ser 460	Gly	Asp	Asn	Leu
Phe 465	Ala	Val	Asp	Val	Arg 0	ily :	Ile .	Asp /	Ala	Glu 475	Glu	Gĵλ.	Arg	Phe	Asr. 480
Asn	Leu	Arg	Leu	Ile 485	Val G	lu A	Arg .	Asn A	Asn 190	Leu	Tyr	Val	Thr	Gly 495	Phe
Val	Asn	Arg	Thr 500	Asn .	Asn V	al P	he '	Tyr # 505	Arg	Phe .	Ala ,	Asp	Phe 510	Ser	His

Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln 535 Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His 550 Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe 565

Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly

Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr 600

Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val 615

Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser 630

Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn 650

Cys His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe 665

Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr His Asn 680

Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met Arg Arg 695

Thr Ile Ser Ser

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2136 base pairs

 - (E) TYPE: nucleic acid(C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (1x) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..2136
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Met	Lys	Ile	Lys	Thr 5	Gly	Ala	CGC	ATC	CTC Leu 10	GCA Ala	TTA Leu	TCC Ser	GCA Ala	TTA Leu 15	ACG Thr	48
ACG	ATG	ATG	TTT	TCC	GCC	TCG	GCT	CTC	GCC	ጸጸጸ	ATC	GAA	GAA	GGT	AAA	96

Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys

CTG GTA ATC TGG ATT AAC GGC GAT AAA GGC TAT AAC GGT CTC GCT GAA Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu

GTC GGT AAG AAA TTC GAG AAA GAT ACC GGA ATT AAA GTC ACC GTT GAG Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu 50 192

CA' Hi: 6	T CCG s Pro	G GA' O Asj	T AA. p Ly:	A CTO	G GAJ J Gli 70	ı Gil	3 AAI 1 Lys	A TT S Ph	c cc e Pr	o Gl	iG GT in Va 5	TT G	CG (GCA Ala	AC Th	T (GC Sly 80	240
GA' Asj	r GG(CCT Pro	T GAO D Asi	C ATT	: Iie	TTC Phe	TGC Tr	G GC.	A CA a Hi 9	s As	C CG p Ar	G T	TT (3GT 31y	GG Gl 9	y T	AC Yr	288
GC: Ala	CAA Glr	A TCT	GG(Gl)	Let	TTC Leu	GCT Ala	GAZ Glu	ATC 1116 105	e Th	c cc r Pr	G GA o As	.C Ai p L	rs A	SCG Ala	TT Ph	c c e G	AG ln	33€
GA(Asp	AAG Lys	CTC Lev 115	ı Tyr	CCC Pro	TTI Phe	ACC Thr	TGG Trp 120	Asp	r GC6	C GT.	A CG l Ar	T T; g T; 13	r A	AC ISD	GG(C A y L	AG ys	384
CTC	Ile 130	ATA	TAC	CCG	ATC Ile	GCT Ala 135	Val	GAA Glu	A GCO	TT:	A TC a Se 14	r Le	G A	TT le	TA: Tyri	A 1	AC sn	432
AAA Lys 145	GAT Asp	CTG Leu	CTG Leu	CCG Pro	AAC Asn 150	CCG Pro	CCA Pro	AAA Lys	ACC Thr	Trp 155	Gl	A GA L Gl	G A' u I	TC le	CCC	G G(la	480
CTG Leu	GAT Asp	AAA Lys	GAA Glu	CTG Leu 165	AAA Lys	GCG Ala	AAA Lys	GGT Gly	Lys 170	Ser	GC(G CT	G A' u Me	TG et	TTC Phe 175	As	AC sn	528
CTG Leu	CAA Gln	GAA Glu	CCG Pro 180	TAC	TTC Phe	ACC Thr	TGG Trp	CCG Pro 185	CTG Leu	ATT	GCT	GC Al	a As	AC Sp 90	GGG Gly	GC G1	ST Y	576
TAT Tyr	GCG Ala	TTC Phe 195	ÀAG Lys	TAT Tyr	GAA Glu	AAC Asn	GGC Gly 200	AAG Lys	TAC Tyr	GAC Asp	ATT	AA: Ly: 20:	s As	AC sp	GTG Val	GG Gl	y C	624
GTG Val	GAT Asp 210	AAC Asn	GCT Ala	GGC Gly	GCG Ala	AAA Lys 215	GCG Ala	GGT Gly	CTG Leu	ACC Thr	TTC Phe 220	Le	G GI u Va	TT d	GAC Asp	CT Le	G u	672
ATT Ile 225	AAA Lys	AAC Asn	AAA Lys	CAC His	ATG Met 230	AAT Asn	GCA Ala	GAC Asp	ACC Thr	GAT Asp 235	TAC Tyr	TC: Ser	TAT	ic (GCA Ala	GA G1 24	u	720
GCT Ala	GCC Ala	TTT Phe	AAT Asn	AAA Lys 245	GGC Gly	GAA Glu	ACA Thr	GCG Ala	ATG Met 250	ACC Thr	ATC Ile	AAC Asn	GG Gl	у	CCG Pro 255	TG(3	768
GCA Ala	TGG	TCC Ser	AAC Asn 260	ATC Ile	GAC Asp	ACC Thr	AGC Ser	AAA Lys 265	GTG Val	AAT Asn	TAT Tyr	GGT Gly	GT. Va. 27	1 7	CG	GT/ Val	A i	816
CTG Leu	CCG Pro	ACC Thr 275	TTC Phe	AAG Lys	GGT Gly	Gln	CCA Pro 280	TCC Ser	AAA Lys	CCG Pro	TTC Phe	GTT Val 285	Gl	c d	TG al	CTC	5 1	864
AGC Ser	GCA Ala 290	GGT Gly	ATT Ile	AAC Asn	Ala .	GCC . Ala . 295	AGT Ser	CCG Pro	AAC Asn	AAA Lys	GAG Glu 300	CTG Leu	GC0 Ala	3 A a L	AA ys	GAC Glu		912
TTC Phe 305	CTC Leu	GAA Glu	AAC Asn	7	CTG Leu : 310	CTG / Leu '	ACT (Thr ,	GAT Asp	GAA Glu	GGT Gly 315	CTG Leu	GAA Glu	GCC Ala	G G a V	al .	AAT Asn 320		960
AAA Lys	GAC Asp	AAA Lys	Pro	CTG (Leu (325	SGT (GCC (Ala N	STA (Val)	4la	CTG Leu 330	AAG Lys	TCT Ser	TAC Tyr	GA0 Glu	ı G	AA lu (35	GAG Glu		1008
TTG Leu	GCG Ala	AAA Lys	GAT Asp	CCA (Pro)	GT / Arg :	ATT (SCC (Ala A	SCC Ala	ACC Thr	ATG Met	GAA Glu	AAC Asn	GCC Ala	G.	AG I	aaa Lys		105€

SUCCED -MIC (1830/0394+ 1 -

			340)				34	5				3 !	50			
GGT Gly	GAA Glu	ATO Ile 355	: Met	CC Pro	G AA	C AT	C CC e Pr 36	G CA o Gli o	G AT n Me	G TC t Se	C GC r Al	T TI a Ph 36	ie Ti	GG T	AT G	CC la	1104
GTG Val	CGT Arg 370		GCG Ala	GT(S ATO	2 AA0 2 Asi 37	n Al	C GCG a Ala	C AG	C GG r Gl	T CG y Ar 38	g Gl	G AC	T G	TC G	AT gp	1152
GAA Glu 385	GCC Ala	CTG Leu	AAA Lys	GA(Asp	GC0 Ala 390	GII	AC:	T TCC r Sea	S AGO	TC: Se: 395	r As	C AA n As	C AA n As	C AA	n As	AT Sn DO	1200
AAC Asn	TAA Asn	AAC Asn	AAC Asn	AAC Asn 405	Lev	GGG Gly	ATO	GAG Glu	GG# Gly 410	/ Arc	F AT	T TC. e Se:	A GA r Gl	A TT u Ph 41	e Ar	g G	1248
GAG Glu	TTT Phe	ACG Thr	ATA Ile 420	GAC Asp	TTT	TCG	ACC Thr	CAA Gln 425	Glr	A AGT	TA	r GTO	TC 1 Se 43	r Se	G TI r Le	A u	1296
AAT Asn	AGT Ser	ATA Ile 435	CGG Arg	ACA Thr	GAG Glu	ATA Ile	TCG Ser 440	ACC	CCT	CTI Leu	GAZ Glu	A CAT His 445	s Ile	A TC	T CA r Gl	.G n	1344
GGG	ACC Thr 450	ACA Thr	TCG Ser	GTG Val	TCT Ser	GTT Val 455	ATT Ile	AAC Asn	CAC His	ACC	CAC His	Gly	AG: Sei	TA:	TT Ph	T e	1392
GCT Ala 465	GTG Val	GAT Asp	ATA Ile	CGA Arg	GGG Gly 470	CTT	GAT Asp	GTC Val	TAT Tyr	CAG Gln 475	GCG Ala	CGT Arg	TTT Phe	GAC Asi	CA: 0 H1: 48:	s	1440
CTT (Arg	rea	тте	11e 485	Glu	Gln	Asn	Asn	Leu 490	Tyr	Val	Ala	Gly	Phe 495	Va.	1	1488
AAT A	1111	^-a	500	ASN	Tnr	Phe	Tyr	Arg 505	Phe	Ser	Asp	Phe	Thr 510	Hıs	Ile	•	1536
TCA (Ser \	va ı	000 Pro 515	GGT Gly	GTG Val	ACA Thr	ACG Thr	GTT Val 520	TCC Ser	ATG Met	ACA Thr	ACG Thr	GAC Asp 525	AGC Ser	AGT Ser	TAT Tyr	-	1584
ACC F Thr 1	ACT Thr :	CT3 Leu	CAA Gln	CGT Arg	Val	GCA Ala 535	GCG Ala	CTG Leu	GAA Glu	CGT Arg	TCC Ser 540	GGA Gly	ATG Met	CAA Gln	ATC Ile		1632
AGT C Ser A 545	rgT (CAC '	TCA (Ser)	CTG Leu	GTT Val 550	TCA Ser	TCA Ser	TAT Tyr	CTG Leu	GCG Ala 555	TTA Leu	ATG Met	GAG Glu	TTC Phe	AGT Ser 560		1680
GGT A	AT ; sn :	ACA (Inr I	wet:	ACC Thr 565	AGA Arg	GAT Asp	GCA Ala	TCC Ser	AGA Arg 570	GCA Ala	GTT Val	CTG Leu	CGT Arg	TTT Phe 575	GTC Val		1728
ACT G Thr V	TC ; al :	::: /	GCA (Ala (580	SAA Slu	GCC Ala	TTA Leu	Arg	TTC Phe 585	AGG Arg	CAG Gln	ATA Ile	CAG Gln	AGA Arg 590	GAA Glu	TTT Phe		1776
CGT C Arg G	TU >	SCA (La 1 SSS	TG T Leu S	CT (GAA . Glu '	Thr .	GCT Ala 600	CCT (Pro	GTG Val	TAT . Tyr :	ACG Thr	ATG Met 605	ACG Thr	CCG Pro	GGA Gly		1824
GAC G Asp V 6	TG G al A 10	sp :	eu 1	ACT (Leu	AAC ' Asn ' 615	TGG Trp	GGG (CGA . Arg	Ile :	AGC Ser 620	AAT Asn	GTG Val	CTT Leu	CCG Pro		1872

GAG Glu 625	Tyr	CGG Arg	GGA Gly	GAG Glu	GAT Asp 630	Gly	GTC Val	AGA Arg	GTG Val	GGG Gly 635	AGA Arg	ATA Ile	TCC Ser	TTT Phe	AAT Asn 640	1920
AAT Asn	ATA Ile	TCA Ser	GCG Ala	ATA Ile 645	CTG Leu	GGG Gly	ACT Thr	GTG Val	GCC Ala 650	GTT Val	ATA Ile	CTG Leu	AAT Asn	TGC Cys 655	CAT His	1968
CAT His	CAG Gln	GGG Gly	GCG Ala 660	CGT Arg	TCT Ser	GTT Val	CGC Arg	GCC Ala 665	GTG Vaļ	AAT Asn	GAA Glu	GAG Glu	AGT Ser 670	CAA Gln	CCA Pro	2016
GAA Glu	TGT	CAG Gln 675	ATA Ile	ACT Thr	GGC Gly	GAC Asp	AGG Arg 680	CCT Pro	GTT Val	ATA Ile	AAA Lys	ATA Ile 685	AAC Asn	AAT Asn	ACA Thr	2064
TTA Leu	TGG Trp 690	GAA Glu	AGT Ser	AAT Asn	ACA Thr	GCT Ala 695	GCA Ala	GCG Ala	TTT Phe	CTG Leu	AAC Asn 700	AGA Arg	AAG Lys	TCA Ser	CAG Gln	2112
			ACA Thr				AT									2136

- (2) INFORMATION FOR SEQ ID NO:35:
 - (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 711 amino acids

 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr

Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys

Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu 35 40

Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu

His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly

Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr

Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gln 105

Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys 115 120 125

Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn 135

Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala

Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn

Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly

Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly 200 Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu 230 Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp 245 Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu 280 . . 285 . Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu 295 Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu 330 Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys 345 Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala 360 Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn 390 Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Arg 405 Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser Ser Leu 4.2.0 Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile Ser Gln 440 Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser Tyr Phe 455 Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe Asp His 470 Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly Phe Val 490 Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr His Ile 505 Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser Ser Tyr 520 Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met Gln Ile 535 Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu Phe Ser 550 Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg Phe Val

				5	65				-	7.0						_	
T:	ır Va	il Ti	hr A			la Le	eu L	ra Di		70 70	-1-	73-	61		57	5 u Phe	
			_	-				51	3 5					590	2		
			, ,				6.0	0					605			o Gly	
Às	p Va 61	1 As 0	sp L	eu T	hr Le	eu As 61	n Tr	rp Gl	у А	rg I	le	Ser 620	Asn	Val	Le	u Pro	>
G1 62	ս Tչ՝ 5	r Ar	g G	ly G	lu As 63	sp Gl	y Va	ıl Ar	g Va	al G 6	1 y 35	Arg	Ile	Ser	Phe	Asn 640	
As	n Il	e Se	r Al	.a II	е Le 5	u Gl	y Th	ır Va	1 A1	la V 50	al	Ile	Leu	Asn	Cys 655	His	
Hi	s Gl:	n Gl	y Al 66	a Ar	g Se	r Va	l Ar	g Al 66	a Va 5	il A	sn (Glu	Glu	Ser 670	Glr	: Pro	
Gl	u Cy:	s Gl 67	n Il 5	e Th	r Gl	y Ası	0 Ar	g Pr	o Va	1 1	le !		Ile 685	Asn	Asn	Thr	
Le	1 Trp	Gl	u Se	r As	n Th	r Ala 695	a Ala	a Al	a Ph	e Le	eu /	Asn 700	Arg	Lys	Ser	Glr	
Phe 705	e Leu	а Ту:	r Th	r Th	r Gl 71	y Lys O	5										
(2)	INE	ORM	LTIO	N FO	R SE	QID	NO : I	36:									
	(ii) MC	(A) (B) (C) (D) (D) (D) (D) (C) (C) (C) (C) (C) (C) (C) (C) (C) (C	LENG TYPE STRAI TOPOI JLE : RE: NAME	TH: : : nuc NDEDI LOGY TYPE:	ACTER 981 b cleic NESS: : lin : DNA CDS 1	ease sir ear (ge	pair id igle									
	(xi					IPTI		SEQ	1 DI	10 : 3	€:						,
ATG Met 1	- 7 - 3	AAG Lys	ACA Thr	GCT Ala	6	GCG Ala	ATT Ile	GCA Ala	GTO Val	Ala	A C' a Le	TG G eu A	CT C	SGT : Sly 1	TTC Phe 15	GCT Ala	
ACC Thr	GTT Val	GCG Ala	CAA Glm 20	Mid	GAC Asp	TAC Tyr	AAG Lys	GAC Asp 25	GAC Asp	GA: Asp	T GA	AC A Sp L	AG A ys L	AG (ys 1 30	ITT .	GAA Glu	!
TTC Phe	AAG Lys	GAA Glu 35	TTT Phe	ACC Thr	TTA Leu	GAC Asp	TTC Phe 40	TCG Ser	ACT Thr	GCA Ala	A AJ B Ly	's T	CG T hr T 45	AT C	TA (GAT Asp	14
TCG Ser	CTG Leu 50	AAT Asn	GTC Val	ATT Ile	cgc Arg	TCT Ser 55	GCA Ala	ATA Ile	GGT Gly	ACT	Pr	A TO LO	TA C. eu G	AG A lr T	CT / hr I	ATT []e	1 9
TCA Ser 65	TCA Ser	GGA Gly	GGT Gly	ACG Thr	TCT Ser 70	TTA Leu	CTG Leu	ATG Met	ATT Ile	GAT Asp 75	Se	T GO r Gl	SC TO	CA G er G	GG (SAT Sp 80	24
AAT Asn	TTG Leu	TTT Phe	GCA Ala	GTT Val 85	GAT Asp	GTC Val	AGA Arg	GGG Gly	ATA Ile 90	GAT Asp	GC Al	A GA a Gl	kg ga .u gj	Lu G	GG C ly A 95	GG Tg	28
TTT	AAT	AAT	CTA	CGG	CTT	ATT	STT	GAA	CGA	AAT	AX		A TA	ντ c.	TG A	.CA	3.3

336

Phe	e As:	n Ası	n Lei 100	n Arg	g Le	ı Ile	e Val	l Glu 109	a Arg	g Ası	n As	n Le	u Ty		.1 Thr	
GGZ Gly	TT:	GT: Val	LASI	AGO Arg	G ACA g Thr	AAI Asn	AAT Asr 120	va]	TTT Phe	TATE Ty:	CG Arg	C TT g Pho 12!	e Al	T GA a As	T TTT p Phe	384
TCA Ser	CAT His	o vai	C ACC	TTT Phe	CCA Pro	GGT Gly 135	Thr	ACA Thr	A GCC Ala	GTT Val	ACI Thi	r Lei	TC Se	T GG r Gl	T GAC y Asp	432
AGT Ser 145	ser	TAT	ACC Thr	ACC Thr	TTA Leu 150	GIn	CGT Arg	GTT Val	GCA Ala	GGG Gly 155	Ile	AGT Ser	CG:	T AC	G GGG r Gly 160	480
ATG Met	CAG Gln	ATA Ile	AAT Asn	CGC Arg 165	HIS	TCG Ser	TTG Leu	ACT Thr	ACT Thr 170	Şer	TAT Tyr	CTG Leu	GA? Asp	TT: Let 175	A ATG	528
TCG Ser	CAT	AGT Ser	GGA Gly 180	Thr	TCA Ser	CTG Leu	ACG Thr	CAG Gln 185	TCT Ser	GTG Val	GCA Ala	AGA Arg	GCC Ala	Met	TTA Leu	576
CGG Arg	TTT Phe	GTT Val 195	ACT Thr	GTG Val	ACA Thr	GCT Ala	GAA Glu 200	GCT Ala	TTA Leu	CGT Arg	TTT Phe	CGG Arg 205	CAA Gln	ATA	CAG Gln	624
AGG Arg	GGA Gly 210	TTT Phe	CGT Arg	ACA Thr	ACA Thr	CTG Leu 215	GAT Asp	GAT Asp	CTC Leu	AGT Ser	GGG Gly 220	CGT Arg	TCT Ser	TAT Tyr	GTA Val	672
ATG Met 225	ACT Thr	GCT Ala	GAA Glu	GAT Asp	GTT Val 230	GAT Asp	CTT Leu	ACA Thr	TTG Leu	AAC Asn 235	TGG Trp	GGA Gly	AGG Arg	TTG Leu	AGT Ser 240	720
AGC Ser	GTC Val	CTG Leu	CCT Pro	GAC Asp 245	TAT Tyr	CAT His	GGA Gly	CAA Gln	GAC Asp 250	TCT Ser	GTT Val	CGT Arg	GTA Val	GGA Gly 255	AGA Arg	768
ATT Ile	TCT Ser	TTT Phe	GGA Gly 260	AGC Ser	ATT Ile	AAT Asn	GCA Ala	ATT Ile 265	CTG Leu	GGA Gly	AGC Ser	GTG Val	GCA Ala 270	TTA Leu	ATA Ile	816
CTG Leu	AAT Asn	TGT Cys 275	CAT His	CAT His	CAT Hıs	Ala	TCG Ser 280	CGA Arg	GTT Val	GCC Ala	AGA Arg	ATG Met 285	GCA Ala	TCT Ser	GAT Asp	864
GAG Glu	TTT Phe 290	CCT Pro	TCT Ser	ATG Met	Cys	CCG Pro 295	GCA Ala	GAT Asp	GGA Gly	Arg	GTC . Val 300	.CGT Arg	GGG Gly	ATT Ile	ACG Thr	912 '
CAC His 305	AAT Asn	AAA Lys	ATA Ile	Leu	TGG Trp 310	GAT ' Asp :	TCA Ser	TCC . Ser	Thr	CTG (Leu (315	GGG Gly	GCA Ala	ATT Ile	CTG Leu	ATG Met 320	960
CGC . Arg .	AGA Arg	ACT Thr	Ile .	AGC Ser 325	AGT ' Ser	TG										981

- (2) INFORMATION FOR SEQ ID NO:37:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 326 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Lys Lys Leu Glu Phe Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly 150 155 Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu 180 Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile 265 Leu Asn Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp 280 Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met . 315 Arg Arg Thr Ile Ser Ser

(2. INFORMATION FOR SEQ ID NO:38:

EDOCIF JAIO GOSDOVANA L.

- (1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 990 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - 'D' TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 1..990

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

1-16	G AAI t Ly:	A AA s Ly	G ACI	ALA	T ATO	C GCC Ala	AT:	r GC e Ala	A GTO a Val	Ala	A CTO	G GC	T GG a Gl	y Ph	C GC: se Ala	T a	48
ACC Thi	GT: Val	r GC0 l Ala	G CAA B Gl: 20	Ala	GAC Asp	TAC Tyr	Lys	G GAG S Asp) Asp	GAT Asp	GAC Asi	AAG Ly:	G AA	s Le	T GAZ u Glu	A. 	96
TTC Phe	CGC Arg	G GAG G Glu 35	ı Pn∈	ACC Thr	ATA	GAC Asp	TTT Phe	e Ser	ACC Thr	CAA Glr	CAA Glr	A AG: Sei 45	Ty:	T GT r Va	C TCT l Ser	-	144
TCC	TTA Leu 50	ASI	AGT Ser	Ile	CGG Arg	ACA Thr 55	GAG Glu	ATA Ile	TCG Ser	ACC Thr	CCI Pro 60	Let	GAJ Glu	A CA 1 Hı	T ATA s Ile	•	192
TCT Ser 65	GII	GGG Gly	ACC Thr	ACA Thr	TCG Ser 70	GTG Val	TCT Ser	GTT Val	ATT Ile	AAC Asn 75	CAC	ACC	CAC His	GG Gl	C AGT Y Ser 80		240
TAT Tyr	TTT Phe	GCT Ala	GTG Val	GAT Asp 85	ATA Ile	CGA Arg	GGG Gly	CTT Leu	GAT Asp 90	GTC Val	TAT Tyr	CAG Gln	GCG Ala	CG: Arg	TTT J Phe		288
GAC Asp	CAT His	CTT	CGT Arg 100	CTG Leu	ATT Ile	ATT Ile	GAG Glu	CAA Gln 105	AAT Asn	AAT Asn	TTA Leu	TAT Tyr	GTG Val 110	Ala	GGG Gly		336
TTC Phe	GTT Val	AAT Asn 115	Thr	GCA Ala	ACA Thr	AAT Asn	ACT Thr 120	TTC Phe	TAC Tyr	CGT Arg	TTT Phe	TCA Ser 125	GAT Asp	TTI Phe	ACA Thr		384
CAT His	ATA Ile 130	TCA Ser	GTG Val	CCC Pro	GGT Gly	GTG Val 135	ACA Thr	ACG Thr	GTT Val	TCC Ser	ATG Met 140	ACA Thr	ACG Thr	GAC Asp	AGC		432
AGT Ser 145	TAT Tyr	ACC Thr	ACT Thr	CTG Leu	CAA Gln 150	CGT Arg	GTC Val	GCA Ala	GCG Ala	CTG Leu 155	GAA Glu	CGT Arg	TCC Ser	GGA Gly	ATG Met 160		480
CAA Gln	ATC Ile	AGT Ser	CGT Arg	CAC His 165	TCA Ser	CTG Leu	GTT Val	TCA Ser	TCA Ser 170	TAT Tyr	CTG Leu	GCG Ala	TTA Leu	ATG Met 175	GAG Glu		528
TTC	AGT Ser	GGT Gly	AAT Asn 180	ACA Thr	ATG Met	ACC Thr	AGA Arg	GAT Asp 185	GCA Ala	TCC Ser	AGA Arg	GCA Ala	GTT Val 190	CTG Leu	CGT Arg		576
TTT Phe	GTC Val	ACT Thr 195	GTC Val	ACA Thr	GCA Ala	Glu.	GCC Ala 200	TTA Leu	CGC Arg	TTC Phe	Arg	CAG Gln 205	ATA Ile	CAG Gln	AGA Arg		624
GAA Glu	TTT Phe 210	CGT Arg	CAG Gln	GCA Ala	Leu	TCT Ser 215	GAA Glu	ACT Thr	GCT Ala	Pro	GTG Val 220	TAT Tyr	ACG Thr	ATG Met	ACG Thr		672
CCG Pro 225	GGA Gly	GAC Asp	GTG Val	Asp	CTC Leu 230	ACT :	CTG Leu	AAC. Asn	Trp (GGG (Gly . 235	CGA . Arg	ATC Ile	AGC Ser	AAT Asn	GTG Val 240		720
CTT Leu	CCG Pro	GAG Glu	TAT Tyr	CGG Arg	GGA (GAG (Glu)	GAT (GGT Gly	GTC Z	AGA (Arg '	STG (GGG . Gly .	AGA Arg	ATA Ile	TCC Ser		76B

				245					250					255		
TTT Phe	AAT Asn	AAT Asn	ATA Ile 260	TCA Ser	GCG Ala	ATA Ile	CTG Leu	GGG Gly 265	ACT Thr	GTG Val	GCC Ala	GTT Val	ATA Ile 170	CTG Leu	AAT Asn	816
TGC Cys	CAT His	CAT His 275	CAG Gln	GGG Gly	GCG Ala	CGT Arg	TCT Ser 280	GTT Val	CGC Arg	GCC Ala	GTG Val	AAT Asn 285	GAA Glu	GAG Glu	AGT Ser	864
CAA Gln	CCA Pro 290	GAA Glu	TGT Cys	CAG Gln	ATA Ile	ACT Thr 295	GGC Gly	GAC Asp	AGG Arg	CCT Pro	GTT Val 300	ATA Ile	AAA Lys	ATA Ile	AAC Asn	912
AAT Asn 305	ACA Thr	TTA Leu	TGG Trp	GAA Glu	AGT Ser 310	AAT Asn	ACA Thr	GCT Ala	GCA Ala	GCG Ala 315	TTT Phe	CTG Leu	AAC Asn	AGA Arg	AAG Lys 320	960
TCA Ser	CAG Gln	TTT Phe	Leu	TAT Tyr 325	ACA Thr	ACG Thr	GGT Gly	AAA Lys	TA 330							990
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(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 329 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala 1 15

Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Lys Lys Leu Glu 20 25 30

Phe Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser 35

Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile 50 55 60

Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser 65 70 75 80

Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe
85 90 95

Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly 100 105 110

Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr 115 120 125

His Ile Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser 130

Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met 145 150 155 160

Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu 165 170 175

Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg 180 185

Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg

		195	;				200)				205	,		
Glu	Phe 210	Arg	Gln	Ala	Leu	Ser 215	Glu	Thr	Ala	Pro	Val 220	Tyr	Thr	Met	Thi
Pro 225	Gly	Asp	Val	Asp	Leu 230	Thr	Leu	Asn	Trp	Gly 235	Arg	Ile	Ser	Asn	Val 240
Leu	Pro	Glu	Tyr	Arg 245	Gly	Glu	Asp	Gly	Val 250	Arg	Val	Gly	Arg	Ile 255	Ser
Phe	Asn	Asn	Ile 260	Ser	Ala	Ile	Leu	Gly 265	Thr	Val	Ala	Val	Ile 270	Leu	Asn
Cys	His	His 275	Gln	Gly	Ala	Arg	Ser 280	Val	Arg	Ala	Val	Asn 285	Glu	Glu	Ser
Gln	Pro 290	Glu	C'ns	Gln	Ile	Thr 295	Gly	Asp	Arg	Prc	Val 300	Ile	Lys	Ile	Asn
Asn 305	Thr	Leu	Trp	Glu	Ser 310	Asn	Thr	Ala	Ala	Ala 315	Phe	Leu	Asn	Arg	Lys 320
Ser	Gln	Phe	Leu	Tyr 325	Thr	Thr	Gly	Lys							

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CLAIMS

What is claimed is:

5 A method of treatment comprising: 1. a) providing: antitoxin directed against at least a portion of an Escherichia coli i) verotoxin in an aqueous solution in therapeutic amount that is administrable. and 10 ii) an intoxicated subject: and administering said antitoxin to said subject. b) The method of Claim 1 wherein said Escherichia coli verotoxin is recombinant. 2. 15 The method of Claim 1 wherein said antitoxin is an avian antitoxin. 3. The method of Claim 2 wherein said recombinant Excherichia coli verotoxin is 4. a fusion protein comprising a non-verotoxin protein sequence and a portion of the Escherichia coli verotoxin VT1 sequence. 20 5. The method of Claim 2 wherein said recombinant Escherichia coli verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the Escherichia coli verotoxin VT2 sequence. 25 The method of Claim 1 wherein said subject is an adult. 6. The method of Claim 1 wherein said subject is a child. 7. The method of Claim 1 wherein said administering is parenteral. 8. 30 9. The method of Claim 1 wherein said administering is oral.

10. A method of prophylactic treatment comprising:

a) providing:

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- i) an antitoxin directed against at least one *Escherichia coli* verotoxin in an aqueous solution in therapeutic amount that is parenterally administrable, and
 - ii) at least one subject is at risk of diarrheal disease; and
- b) parenterally administering said antitoxin to said subject.
- 11. The method of Claim 10, wherein said subject is at risk of developing extraintestinal complications of *Escherichia coli* infection.
 - 12. The method of Claim 11, wherein said extra-intestinal complication is hemolytic uremic syndrome.
- 15 13. A composition comprising neutralizing antitoxin directed against at least one Escherichia coli verotoxin in an aqueous solution in therapeutic amounts.
 - 14. The composition of Claim 13 wherein said *Escherichia coli* verotoxin is a recombinant toxin.
 - 15. The composition of Claim 14 wherein said recombinant Escherichia coli verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the Escherichia coli verotoxin VT1 sequence.
- 25 16. The composition of Claim 14 wherein said recombinant Escherichia coli verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the Escherichia coli verotoxin VT2 sequence.
- 17. The composition of Claim 14 wherein said antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin.
 - 18. The composition of Claim 14 wherein said portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT1.

19. The composition of Claim 14 wherein said portion of Escherichia coli is selected from the group consisting of subunit A and subunit B of VT2.

- 20. The composition of Claim 14 wherein said antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin.
 - 21. The composition of Claim 14 wherein said antitoxin is an avian antitoxin.
 - 22. A method of treatment of enteric bacterial infections comprising:
 - a) providing:

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- i) an avian antitoxin directed against at least one verotoxin produced by Escherichia coli in an aqueous solution in therapeutic amount that is parenterally administrable, and
 - ii) at least one infected subject; and
- b) parenterally administering said avian antitoxin to said subject.
- 23. The method of Claim 18 wherein said *Escherichia coli* is selected from the group consisting of *Escherichia coli* serotypes O157:H7. O1:NM: O2:H5: O2:H7: O4:NM: O4:H10: O5:NM: O5:H16: O6:H1: O18:NM: O18:H7; O25:NM: O26:NM: O26:H11: O26:H32: O38:H21: O39:H4: O45:H2: O50:H7: O55:H7: O55:H10: O82:H8: O84:H2: O91:NM: O91:H21: O103:H2: O111:NM: O111:H8: O111:H30: O111:H34: O113:H7: O113:H21: O114:H48: O115:H10: O117:H4: O118:H12: O118:H30: O121:NM: O121:H19: O125:NM: O125:H8: O126:NM: O126:H8: O126:NM: O126:H8: O128:NM: O128:H8: O128:H12:
- O128:H25: O145:NM: O125:H25: O146:H21: O153:H25: O157:NM: O163:H19: O165:NM:
- 25 O165:19: and O165:H25
 - 24. The method of Claim 22 wherein said antitoxin comprises antitoxin directed against at least one *Escherichia coli* verotoxin.
- 30 25. The method of Claim 22 wherein said antitoxin is cross-reactive with at least one Escherichia coli verotoxin.

26. The method of Claim 22 wherein said antitoxin is reactive against toxins produced by members of the genus Shigella.

- 27. The method of Claim 26, wherein said antitoxin is reactive against toxins produced by Shigella dysenteriae.
 - 28. A method for detecting Escherichia coli verotoxin in a sample comprising:
 - a) providing:

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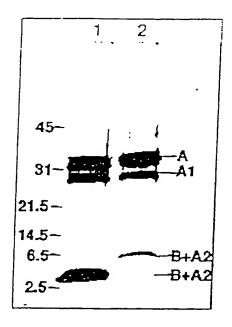
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- i) a sample:
- ii) an antitoxin raised against Escherichia coli verotoxin; and
- iii) a reporter reagent capable of binding said antitoxin; and
- b) adding said antitoxin to said sample so that said antitoxin binds to the Escherichia coli verotoxin in said sample.
- 29. The method of Claim 28, wherein said antitoxin is an avian antitoxin.
- 30. The method of Claim 28, further comprising the steps of:
 - c) washing said unbound antitoxin from said sample:
- d) adding said reporter reagent to said sample so that said reporter reagent binds to said bound antitoxin:
 - e) washing said unbound reporter reagent from said sample; and
- f) detecting said reporter reagent bound to said antitoxin bound to the Escherichia coli verotoxin so that the verotoxin is detected.
- 25 31. The method of Claim 30 wherein said detecting is selected from the group consisting of enzyme immunoassay, radioimmunoassay, fluorescence immunoassay, flocculation, particle agglutination, and *in situ* chromogenic assay.
 - 32. The method of Claim 30 wherein said sample is a biological sample.
 - 33. The method of Claim 30 wherein said sample is an environmental sample.

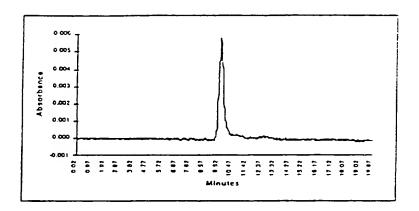
Figure 1. SDS-PAGE of rVT1 and rVT2



rVT1 (Lane 1) and rVT2 (Lane 2). Positions of molecular weight markers (Kda) are shown at the left. VT component polypeptides are identified at the right.

Figure 2.

HPLC of rVT1



HPLC of rVT2

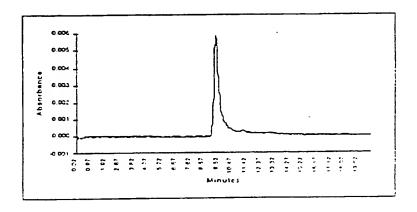


Figure 3. rVT1 and rVT2 Toxicity in Vero Cell Culture

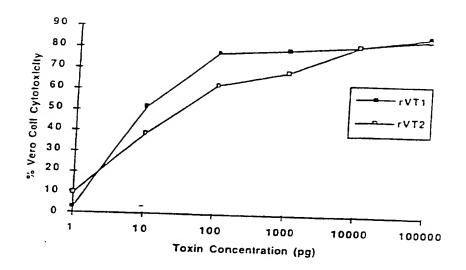
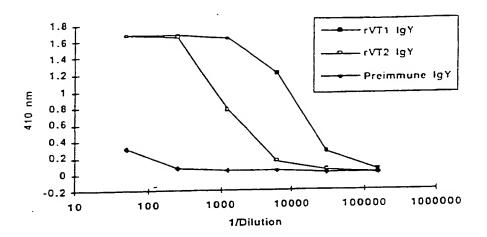


Figure 4. • ELA Reactivity of rVT1 and rVT2 Antibodies to rVT1



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Figure 5. EIA Reactivity of rVT1 and rVT2 Antibodies to rVT2

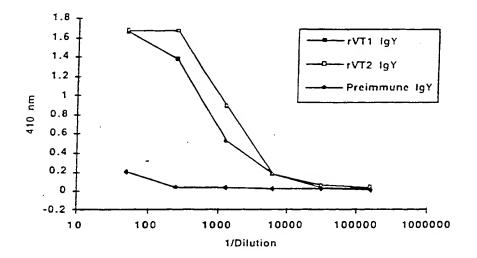
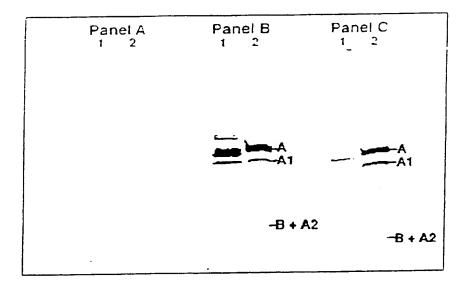


Figure 6.
Western Blot Reactivity of rVT1 and rVT2 Antibodies to rVT's



In this Figure, Panel A contains preimmune IgY, Panel B contains rVT1 IgY, and Panel C contains rVT2 IgY. Lane 1 in each panel contains rVT1 ($2\mu g$) and Lane 2 contains rVT2 ($2\mu g$).

Figure 7.

Neutralization of rVT1 Cytotoxicity in Vero Cells

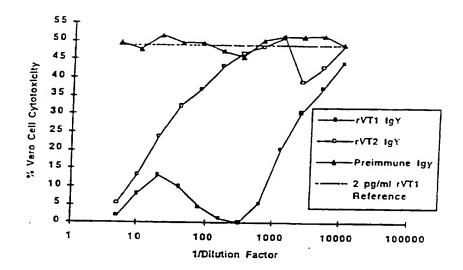


Figure 8.

Neutralization of rVT2 Cytotoxicity in Vero Cells

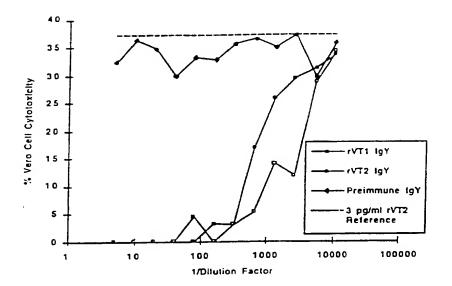
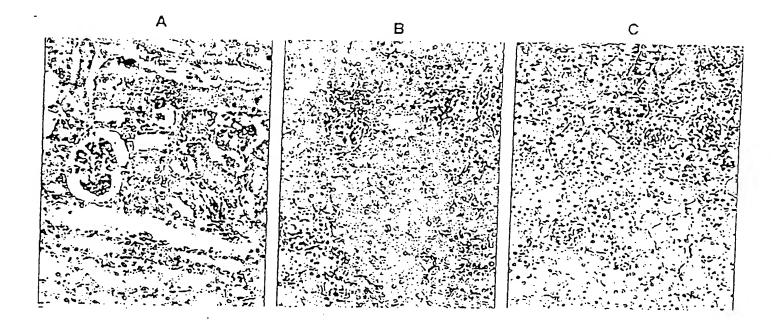


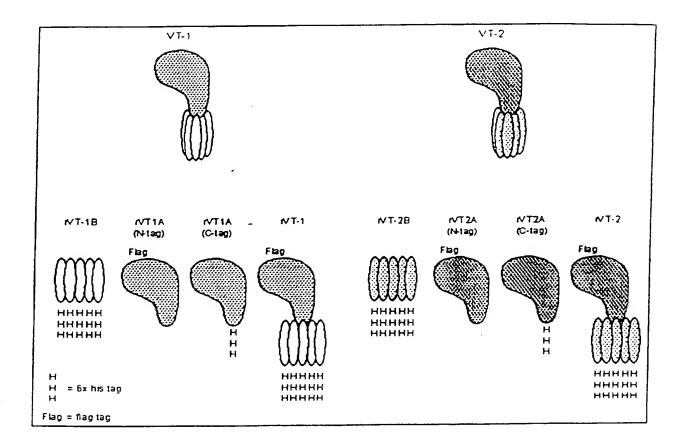
Figure 9.

Renal Sections from E. coli O157:H7-Infected Mice Treated with 1gY



Representative kidney sections from mice treated with preimmune (Panel A), rVT1 (Panel B) or rVT2 (Panel C) lgY 4 hrs. after infection.

Figure 10.
Fusion Constructs of VT Components and Affinity Tags



INTERNATIONAL SEARCH REPORT

International application No. PCT/US96/04093

] A. Cl.	ASSIFICATION OF SUBJECT MATTER		
IPC(6)	A01K 39/00, 39/02; G01N 35/537		
US CL	Please See Extra Sheet		
According	to International Patent Classification (IPC) or to both	r national classification and IPC	
B. FIE	LDS SEARCHED		
Minmum	documentation searched (classification system follower	d by classification symbols.	
U.S. :	424/134.1, 141.1, 150.1, 157.1, 164.1, 169.1, 192, 542, 543-547	1 200 1 236 1 241 1 907 404 505	
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C. 1/()(UMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where ap	propriate, of the relevant passages	Relevant to claim No
Υ	BOYD at all Saralasian Barre		
·	BOYD et al. Serological Responses	to the B Subunit of Shiga-	1-33
	Like Toxin 1 and Its Peptide Fragr	nents Indicate that the B	
	Subunit Is a Vaccine Candidate To	Counter the Action of the	
	Toxin. Infection and Immunity. Ma pages 750-757.	rch 1991, Vol. 59, No. 3,	
	pages 750-757.		
Y	US 5,326,559 A (MILLER) 05 July	1004	
	TO STOLET, SSS A (MILLERY) OS July	1994, columns 4-7.	1-33
X	US 5,164,298 A (LINGWOOD et	all 17 November 1992	20 20 01 00
	columns 10-13.	a., ., November 1992,	28, 30, 31, 32, 33
Y			33
]			1-27 and 29
			1°27 and 29
Υ	US 4,748,018 A (STOLLE et al) 3	1 May 1988, column 4	3, 21, 22, 29
	lines 25-55.	, , , , , , , , , , , , , , , , , , , ,	0, 21, 22, 25
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INTERNATIONAL SEARCH REPORT

International application No. PCT/US96/04093

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	US 4,550,019 A (POLSON) 29 October 1985, column 4, lines 46-68.	3, 21, 22, 29
,	US 5,204,097 A (ARNON et al) 20 April 1993, column 2, lines 1-16, column 3, lines 33-56 and column 5, lines 53-67.	2 and 14
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INTERNATIONAL SEARCH REPORT

International application No. PCT/US96/04093

A	CLASSIFICATION	ΟF	SUBJECT	MATTER.
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424/134.1, 141.1, 150.1, 157.1, 164.1, 169.1, 192.1, 200.1, 236.1, 241.1, 801, 804, 809, 826; 435/7.37; 436/538, 542, 543-547

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS, MEDLINE, APS

search terms; verotoxin, verocytoxin, shiga, rvt1, rvt2, rslt1 or rslt2, vaccin? or treat?, recombinant

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(54) Title: TREATMENT FOR VEROTOXIN-PRODUCING ESCHERICHIA COLI

(57) Abstract

The present invention includes methods for generating neutralizing antitoxin directed against verotoxins. In particular, the antitoxin directed against these toxins is produced in avian species using soluble recombinant verotoxin proteins. This avian antitoxin is designed so as to be administrable in therapeutic amounts and may be in any form (i.e., as a solid or in aqueous solution). These antitoxins are useful in the treatment of humans and other animals intoxicated with at least one bacterial toxin, as well as for diagnostic assays to detect the presence of toxin in a sample.

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TREATMENT FOR VEROTOXIN-PRODUCING ESCHERICHLA COLI

FIELD OF THE INVENTION

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The present invention relates to antitoxin therapy for humans and other animals, and diagnostic assays to detect toxins. Antitoxins which neutralize the pathologic effects of *Escherichia coli* toxins, such as verotoxin are provided.

BACKGROUND OF THE INVENTION

A. Escherichia coli as a Pathogenic Organism

Escherichia coli is the organism most commonly isolated in clinical microbiology laboratories, as it is usually present as normal flora in the intestines of humans and other animals. However, it is an important cause of intestinal, as well as extraintestinal infections. For example, in a 1984 survey of nosocomial infections in the United States. *E. coli* was associated with 30.7% of the urinary tract infections. 11.5% of the surgical wound infections. 6.4% of the lower respiratory tract infections. 10.5% of the primary bacteremia cases. 7.0% of the cutaneous infections, and 7.4% of the other infections (J.J. Farmer and M.T. Kelly, "Enterobacteriaceae." *in* Manual of Clinical Microbiology, Balows *et al.*(eds). American Society for Microbiology, [1991], p. 365). Surveillance reports from England. Wales and Ireland for 1986 indicate that *E. coli* was responsible for 5.473 cases of bacteremia (including blood, bone marrow, spleen and heart specimens): of these, 568 were fatal. For spinal fluid specimens, there were 58 cases, with 10 fatalities (J.J. Farmer and M.T. Kelly, "Enterobacteriaceae." *in* Manual of Clinical Microbiology, Balows *et al.*(eds). American Society for Microbiology, [1991], p. 366). There are no similar data for United States, as these are not reportable diseases in this country.

Studies in various countries have identified certain serotypes (based on both the O and H antigens) that are associated with the four major groups of *E. coli* recognized as enteric pathogens. Table 1 lists common serotypes included within these groups. The first group includes the classical enteropathogenic serotypes ("EPEC"): the next group includes those that produce heat-labile or heat-stable enterotoxins ("ETEC"): the third group includes the enteroinvasive strains ("EIEC") that mimic *Shigella* strains in their ability to invade and multiply within intestinal epithelial cells; and the fourth group includes strains and serotypes that cause hemorrhagic colitis or produce Shiga-like toxins (or verotoxins) ("VTEC" or "EHEC" [enterohemmorrhagic *E. coli*]).

Table 1.
Pathogenic E. coli Serotypes

Group	Associated Serotypes
Enterotoxigenic (ETEC)	O6:H16: O8:NM; O8:H9: O11:H27; O15:H11: O20:NM: O25:NM: O25:H42: O27:H7; O27:H20; O63:H12: O78:H11: O78:H12: O85:H7: O114:H21; O115:H21: O126:H9: O128ac:H7: O128ac:H12: O128ac:H21; O148:H28: O149:H4: O159:H4: O159:H20: O166:H27; and O167:H5
Enteropathogenic (EPEC)	O26:NM: O26:H11; O55:NM: O55:H6: O86:NM: O86:H2; O86:H34; O111ab:NM; O111ab:H2: O111ab:H12: O111ab:H21: O114:H2: O119:H6; O125ac:H21: O127:NM: O127:H6: O127:H9: O127:H21: O128ab:H2: O142:H6: and O158:H23
Enteroinvasive (EIEC)	O28ac:NM: O29:NM; O112ac:NM: O115:NM: O124:NM: O124:H7: O124:H30; O135:NM: O136:NM: O143:NM: O144:NM: O152:NM: O164:NM: and O167:NM
Verotoxin-Producing (VTEC))	O1:NM: O2:H5: O2:H7; O4:NM: O4:H10: O5:NM: O5:H16: O6:H1: O18:NM; O18:H7: O25:NM: O26:NM: O26:H11: O26:H32: O38:H21; O39:H4; O45:H2: O50:H7: O55:H7: O55:H10: O82:H8; O84:H2: O91:NM; O91:H21: O103:H2: O111:NM; O111:H8: O111:H30: O111:H34: O113:H7: O113:H21: O114:H48: O115:H10; O117:H4; O118:H12: O118:H30: O121:NM: O121:H19; O125:NM: O125:H8; O126:NM: O126:H8: O128:NM: O128:H2: O128:H8: O128:H12; O128:H25; O145:NM: O125:H25: O146:H21: O153:H25; O157:NM; O157:H7; O163:H19: O165:NM: O165:19: and O165:H25

B. Verotoxin Producing Strains of E. coli

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Although all of these disease-associated serotypes cause potentially life-threatening disease. *E. coli* O157:H7 and other verotoxin-producing strains have recently gained widespread public attention in the United States due to their recently recognized association with two serious extraintestinal diseases, hemolytic uremic syndrome ("HUS") and thrombotic thrombocytopenic purpura ("TTP"). Worldwide, *E. coli* O157:H7 and other verotoxin-producing *E. coli* (VTEC) are an increasingly important human health problem. First identified as a cause of human illness in early 1982 following two outbreaks of food-related hemorrhagic colitis in Oregon and Michigan (M.A. Karmali, "Infection by Verocytotoxin-Producing *Escherichia coli*," Clin. Microbiol. Rev., 2:15-38 [1989]; and L. W. Riley, *et al.* "Hemorrhagic colitis associated with a rare *Escherichia coli* serotype," New Eng. J. Med.,

308: 681-685 [1983]), the reported incidence of VTEC-associated disease has risen steadily, with outbreaks occurring in the U.S., Canada, and Europe.

With increased surveillance. *E. coli* O157:H7 has been recognized in other areas of the world including Mexico. China. Argentina. Belgium. and Thailand (N. V. Padhye and M. P. Doyle. "*Escherichia coli* O157:H7: Epidemiology, pathogenesis and methods for detection in food." J. Food. Prot., 55: 555-565 [1992]: and P. M. Griffin and R. V. Tauxe. "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome." Epidemiol. Rev., 13: 60 [1991]).

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The disease attracted national attention in the U.S. after a major outbreak in the Pacific Northwest that was associated with consumption of undercooked E. coli O157:H7contaminated hamburgers. Over 700 hundred people fell ill (more than 170 were hospitalized) and four young children died (P. Recer, "Experts call for irradiation of meat to protect against food-borne bacteria." Associated Press. 7/12/94 [1994]). Several outbreaks since then have underscored the potential severity and multiple mechanisms for transmission of VTEC-associated diseases (M. Bielaszewská et al., "Verotoxigenic (enterohaemorrhagic) Escherichia coli in infants and toddlers in Czechoslovakia." Infection 18: 352-356 [1990]: A. Caprioli et al.. "Hemolytic-uremic syndrome and Vero cytotoxin-producing Escherichia coli infection in Italy, "J. Infect. Dis., 166: 184-158 [1992]; A. Caprioli, et al., "Community-wide Outbreak of Hemolytic-Uremic Syndrome Associated with Non-O157 Verocytotoxin-Producing Escherichia coli," J. Infect. Dis., 169: 208-211 [1994]; N. Cimolai, "Low frequency of high level Shiga-like toxin production in enteropathogenic Escherichia coli serogroups." Eur. J. Pediatr., 151: 147 [1992]: and R. Voelker., "Panel calls E. coli screening inadequate." Escherichia coli O157:H7--Panel sponsored by the American Gastroenterological Association Foundation in July 1994, Medical News & Perspectives, J. Amer. Med. Assoc., 272: 501 [1994]).

While O157:H7 is currently the predominant *E. coli* serotype associated with illness in North America, other serotypes (as shown in Table 1, and in particular O26:H11, O113:H21, O91:H21 and O111:NM) also produce verotoxins which appear to be important in the pathogenesis of gastrointestinal manifestations and the hemolytic uremic syndrome (P. M. Griffin and R. V. Tauxe. "The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome." Epidemiol. Rev., 13: 60 [1990]; M. M. Levine, *et al.*, "Antibodies to Shiga holotoxin and to two synthetic peptides of the B subunit in sera of patients with *Shigelia dysenteriae* 1

dysentery." J. Clin. Microbiol.. 30: 1636-1641 [1992]; and C. R. Dorn. et al., "Properties of Vero cytotoxin producing Escherichia coli of human and animal origin belonging to serotypes other than O157:H7," Epidemiol. Infect.. 103: 83-95 [1989]). Since organisms with these serotypes have been shown to cause illness in humans they may assume greater public health importance over time (P. M. Griffin and R. V. Tauxe. "The epidemiology of infections caused by Escherichia coli O157:H7, other enterohemorrhagic E. coli. and the associated hemolytic uremic syndrome." Epidemiol. Rev., 13: 60 [1990]).

Clinicians usually observe cases of hemolytic uremic syndrome ("HUS") clustered in a geographic region. However, small outbreaks are likely to be missed because many laboratories do not routinely screen stool specimens for *E. coli* O157:H7. Many cases related to non-commercial food preparation also probably go unrecognized. Nonetheless, *E. coli* O157:H7 is responsible for a large number of cases, as more than 20,000 cases of *E. coli* O157:H7 infection are reported annually in the U.S., with 400–500 deaths from HUS. However, these estimates were compiled when only 11 states mandated reporting of *E. coli* O157:H7. Twenty-nine states have recently made *E. coli* O157:H7 intection a reportable disease (R. Voelker, "Panel calls *E. coli* screening inadequate: *Excherichia coli* O157:H7: panel sponsored by the American Gastroenterological Association Foundation in July 1994. Medical News & Perspectives." J. Amer. Med. Assoc., 272: 501 [1994]). Indeed, the Centers for Disease Control recently added *E. coli* O157:H7 to their list of reportable diseases ("Public Health Threats." Science 267:1427 [1995]).

C. Nature of Verotoxin-Induced Disease

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Risk factors for HUS progression following infection with *E. coli* O157:H7 include age (very young or elderly), bloody diarrhea, leukocytosis, fever, large amounts of ingested pathogen, previous gastrectomy, and the use of antimicrobial agents (in particular, trimethoprim-sulfamethoxazole)(A. A. Harris *et al.*, "Results of a screening method used in a 12 month stool survey for *Escherichia coli* O157:H7." J. Infect. Dis., 152: 775-777 [1985]; and M. A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*," Clin. Microbiol. Rev., 2: 15-38 [1989]).

As indicated above. *E. coli* O157:H7 is associated with significant morbidity and mortality. The spectrum of illness associated with *E. coli* O157:H7 infection includes asymptomatic infection, mild uncomplicated diarrhea, hemorrhagic colitis. HUS, and TTP". Hemorrhagic colitis (or "ischemic colitis") is a distinct clinical syndrome characterized by

sudden onset of abdominal cramps—likened to the pain associated with labor or appendicitis—followed within 24 hours by watery diarrhea. One to two days later, the diarrhea turns grossly bloody in approximately 90% of patients and has been described as "all blood and no stool" (C. H. Pai et al., "Sporadic cases of hemorrhagic colitis associated with Escherichia coli O157:H7," Ann. Intern. Med., 101: 738-742 [1984]: and R. S. Remis et al., "Sporadic cases of hemorrhagic colitis associated with Escherichia coli O157:H7," Ann. Intern. Med., 101: 738-742 [1984]). Vomiting may occur, but there is little or no fever. The time from ingestion to first loose stool ranges from 3–9 days (with a mean of 4 days) L. W. Riley et al., "Hemorrhagic colitis associated with a rare Escherichia coli serotype," New Eng. J. Med., 308: 681-685 [1983]: and D. Pudden et al., "Hemorrhagic colitis in a nursing home," Ontario Can. Dis. Weekly Rpt., 11: 169-170 [1985]), and the duration of illness ranges generally from 2–9 days (with a mean of 4 days).

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HUS is a life-threatening blood disorder that appears within 3--7 days following onset of diarrhea in 10-15% of patients. Those younger than 10 years and the elderly are at particular risk. Symptoms include renal glomerular damage, hemolytic anemia (rupturing of erythrocytes as they pass through damaged renal glomeruli), thrombocytopenia and acute kidney failure. Approximately 15% of patients with HUS die or suffer chronic renal failure. Indeed, HUS is a leading cause of renal failure in childhood (reviewed by M.A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*," Clin. Microbiol. Rev., 2: 15-38 [1989]). Currently, blood transfusion and dialysis are the only therapies for HUS.

TTP shares similar histopathologic findings with HUS, but usually results in multiorgan microvascular thrombosis. Neurological signs and fever are more prominent in TTP, compared with HUS. Generally occurring in adults. TTP is characterized by microangiopathic hemolytic anemia, profound thrombocytopenia, fluctuating neurologic signs, fever and mild azotemia (H. C. Kwaan, "Clinicopathological features of thrombotic thrombocytopenic purpura," Semin, Hematol., 24: 71-81 [1987]; and S. J. Machin, "Clinical annotation: Thrombotic thrombocytopenic purpura," Br. J. Hematol., 56: 191-197 [1984]). Patients often die from microthrombi in the brain. In one review of 271 cases, a rapidly progressive course was noted, with 75% of patients dying within 90 days (E.L. Amorosi and J.E. Ultmann, "Thrombotic thrombocytopenic purpura: Report of 16 cases and review of the literature," Med., 45:139-159 (1966).

Other diseases associated with E. coli O157:H7 infection include hemorrhagic cystitis and balantitis (W. R. Grandsen et al., "Hemorrhagic cystitis and balantitis associated with

verotoxin-producing Escherichia coli O157:H7." Lancet ii: 150 [1985]), convulsions, sepsis with other organisms and anemia (P. C. Rowe et al., "Hemolytic anemia after childhood Escherichia coli O157:H7 infection: Are females at increased risk?" Epidemiol. Infect., 106: 523-530 [1991]).

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D. Mechanism of Pathogenesis

Verotoxins are strongly linked to *E. coli* O157:H7 pathogenesis. All clinical isolates of *E. coli* O157:H7 have been shown to produce one or both verotoxins (VT1 and VT2) (C. A. Bopp *et al.*, "Unusual Verotoxin-producing *Escherichia coli* associated with hemorrhagic colitis," J. Clin. Microbiol., 25: 1486-1489 [1987]). Both of these toxins are cytotoxic to Vero (African green monkey kidney) and HeLa cells. and cause paralysis and death in mice (A. D. O'Brien *et al.*, "Purification of *Shigella dysenteriae* 1 (Shiga) like toxin from *Escherichia coli* O157:H7 strain associated with hemorrhagic colitis," *Luncet* ii: 573 [1983]). These toxins are sometimes referred to in the literature as Shiga-like toxins I and II (SLT-I and SLT-II, respectively), due to their similarities with the toxins produced by *Shigella*. Indeed, much of our understanding of *E. coli* VTs is based on information accumulated on Shiga toxins. Shiga toxin, first described in 1903, has been recognized as one of the most potent bacterial toxins for eukaryotic cells (reviewed by M.A. Karmali, "Infection by Verocytotoxin-producing *Escherichia coli*," Clin, Microbiol, Rev., 2: 15-38 [1989]). Hereinafter, the VT convention will be used; thus, VT1 and VT2 correspond to SLT-I and SLT-II, respectively.

While the pathogenic mechanism of *E. coli* O157:H7 infection is incompletely understood, it is believed that ingested organisms adhere to and colonize the intestinal mucosa, where toxins are released which cause endothelial cell damage and bloody diarrhea. It is also postulated that hemorrhagic colitis progresses to HUS when verotoxins enter the bloodstream, damaging the endothelial cells of the microvasculature and triggering a cascade of events resulting in thrombus deposition in small vessels. These microthrombi occlude the microcapillaries of the kidneys (particularly in the glomeruli) and other organs, resulting in their failure (J. J. Byrnes and J. L. Moake, "TTP and HUS syndrome: Evolving concepts of pathogenesis and therapy." Clin. Hematol., 15: 413-442 [1986]; and T. G. Cleary, "Cytotoxin-producing *Escherichia coli* and the hemolytic uremic syndrome," Pediatr. Clin. North Am., 35: 485-501 [1988]). Verotoxins entering the bloodstream may also result in direct kidney cytotoxicity.

VT1 is immunologically and structurally indistinguishable from Shiga toxin produced by Shigella dysemeriae (A. D. O'Brien et al., "Purification of Shigella dysemeriae 1 (Shiga) like toxin from Escherichia coli O157:H7 strain associated with hemorrhagic colitis." Lancei ii: 573 [1983]). VT1 and VT2 holotoxins each consist of one A and five B subunits (A. Donohue-Rolfe et al., "Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross reactive monoclonal antibodies." Infect. Immun., 57: 3888-3893 [1989]; and A. Donohue-Rolfe et al., "Simplified high yield purification of Shigella toxin and characterization of subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies." J. Exp. Med., 160: 1767-1781 [1984]). The toxic A subunit is enzymatically active, while the B subunit binds the holotoxin to the receptor on the target eukaryotic cell.

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Crystal structure analysis of Shiga holotoxin and VT1 B subunit pentamers have shown that the holotoxin assembles with the C-terminal end of the A subunit associating with, and inserting within, a pentamer of B chains (P. E. Stein et al., "Crystal structure of the cell-binding B oligomer of verotoxin-1 from E. coli," Nature 355: 748-750 [1902], and M.E. Fraser et al., "Crystal structure of the holotoxin from Shigella desemerate at 2.5 Å resolution," Struct. Biol., 1:59-64 [1994]). This conformation is consistent with the observation that a C-terminally truncated A1 subunit of VT1 is toxic (in a ribosomal inhibition assay), but cannot associate with B subunit pentamers (P. R. Austin et al., "Evidence that the A₂ fragment of Shiga-like toxin type I is required for holotoxin integrity," Infect. Immun., 62: 1768 [1994]).

The Verotoxin A Subunit. Examination of the crystal structure of Shiga holotoxin indicates that the N-terminus of its A subunit is both surface-exposed and functionally important. Removal of amino acid interval 3–18 of the A subunit completely abolished toxicity (L. P. Perera et al., "Mapping the minimal contiguous gene segment that encodes functionally active Shiga-like toxin II." Infect. Immun., 59: 829-835 [1991]) while removal of interval 25–44 retained toxicity but abolished its association with B subunit pentamers (J. E. Haddad et al., "Minimum domain of the Shiga toxin A subunit required for enzymatic activity," J. Bacteriol., 175: 4970-4978 [1993]). Deletion of the first 13 residues of the homologous ricin A subunit also abolished toxicity, while deletion of the first 9 residues did not (M. J. May, et al., "Ribosome inactivation by ricin A chain: A sensitive method to assess the activity of wild-type and mutant polypeptides." EMBO J., 8: 301-308 [1989]).

The Verotoxin B Subunit. Studies of Shiga toxin B subunit suggest that neutralizing epitopes may also be present at both the N- and C-terminal regions of VT1 and VT2 B

subunits. Polyclonal antibodies raised against peptides from these regions (residues 5–18. 13–26. 7–26. 54–67 and 57–67) show partial neutralization of Shiga toxin (I. Harari and R. Arnon. "Carboxy-terminal peptides from the B subunit of Shiga toxin induce a local and parenteral protective effect," Mol. Immunol.. 27: 613-621 [1990]: and I. Harari *et ai.*. "Synthetic peptides of Shiga toxin B subunit induce antibodies which neutralize its biological activity." Infect. Immun., 56: 1618-1624 [1988]). Deletion of the last five amino acids of Shiga toxin B (M. P. Jackson *et al.*. "Functional Analysis of the Shiga toxin and Shiga-like toxin Type II variant binding subunits by using site-directed mutagenesis." J. Bacteriol., 172: 653-658 [1990]), or four amino acids of VT2 B (L. P. Perera *et al.*. "Mapping the minimal contiguous gene segment that encodes functionally active Shiga-like toxin II." Infect. Immun., 59: 829-835 [1991]), eliminate toxin activity, while deletion of the last two amino acids of VT2 B subunit reduced cytotoxicity. In contrast, the addition of an 18 or 21 amino acid extension to the native C-terminus of the VT2 B subunit was presumably conformationally correct, as these proteins assembled cytotoxic holotoxin.

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Various approaches to express recombinant verotoxins have included individual or coordinate expression of A and B subunits from high-copy number plasmids and expression with fusion partners (J. E. Haddad et al., "Minimum domain of the Shiga toxin A subunit required for enzymatic activity." J. Bacteriol., 175: 4970-4978 : J. E. Haddad, and M. P. Jackson. "Identification of the Shiga toxin A-subunit residues required for holotoxin assembly." J. Bacteriol., 175: 7652-7657 [1993]: M. P. Jackson et al., "Mutational analysis of the Shiga toxin and Shiga-like toxin II enzymatic subunits." J. Bacteriol., 172: 3346-3350 [1990]: C. J. Hovde et al.. "Evidence that glutamic acid 167 is an active-site residue of Shigalike toxin I." Proc. Natl. Acad. Sci., 85: 2568-2572 [1988]: R. L. Deresiewicz et al., "The role of tyrosine-114 in the enzymatic activity of the Shiga-like toxin I A-chain." Mol. Gen. Genet., 241: 467-473 [1993]; T. M. Zollman et al., "Purification of Recombinant Shiga-like Toxin Type I A, Fragment from Escherichia coli." Protein Express.Purific., 5: 291-295 [1994]: K. Ramotar. et al.. "Characterization of Shiga-like toxin I B subunit purified from overproducing clones of the SLT-I B cistron." Biochem J., 272: 805-811 [1990]: S. B. Calderwood et al.. "A system for production and rapid purification of large amounts of the Shiga toxin/Shiga-like toxin I B subunit," Infect. Immun., 58: 2977-2982 [1990]; D. W. K. Acheson. et al.. "Comparison of Shiga-like toxin I B-subunit expression and localization in Escherichia coli and Vibrio cholerae by using trc or Iron-regulated promoter systems." Infect. Immun. 61: 1098-1104 [1993]; M. P. Jackson et al., "Nucleotide sequence analysis and

comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933," FEMS Microbiol. Lett., 44: 109-114 [1987]: J. W. Newland *et al.*, "Cloning of genes for production of *Escherichia coli* Shiga-like toxin type II." Infect. Immun. 55: 2675-2680 [1987]; and F. Gunzer and H. Karch. "Expression of A and B subunits of Shiga-like toxin II as fusions with glutathione *S*-transferase and their potential for use in seroepidemiology." J. Clin. Microbiol., 31: 2604-2610 [1993]: and D.W. Acheson *et al.*, "Expression and purification of Shiga-like toxin II B subunits." Inf. Immun., 63:301-308 [1995]). In one case, bench top fermentation techniques yielded 22 mg/liter of soluble recombinant protein (D. W. K. Acheson, *et al.*, "Comparison of Shiga-like toxin I B-subunit expression and localization in *Escherichia coli* and *Vibrio cholerae* by using *trc* or Iron-regulated promoter systems." Infect. Immun. 61: 1098-1104 [1993]). However, there have been no systematic approaches to identifying the appropriate spectrum of VT antigens. preserving immunogen and immunoabsorbant antigenicity and scaling-up.

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The receptor for VT1 and VT2 is a globotriaosyl ceramide containing a galactose α-(1-4)- galactose-β-(1-4) glucose ceramide (Gb3) (C. A. Lingwood *et al.*. "Glycolipid binding of natural and recombinant *Escherichia coli* produced verotoxin *in vitro*," J. Biol. Chem., 262: 1779-1785 [1987]; and T. Wadell *et al.*. "Globotriaosyl ceramide is specifically recognized by the *Escherichia coli* verocytotoxin 2." Biochem. Biophys. Res. Commun., 152: 674-679 [1987]). Gb3 is abundant in the cortex of the human kidney and is present in primary human endothelial cell cultures. Hence, the identification of Gb3 as the functional receptor for VT1 and VT2 is consistent with their role in HUS pathogenesis, in which endothelial cells of the renal vasculature are the principal site of damage. Therefore, toxin-mediated pathogenesis may follow a sequence of B subunit binding to Gb3 receptors on kidney cells, toxin internalization, enzymatic reduction of the A subunit to an A1 fragment, binding of the A1 subunit to the 60S ribosomal subunit, inhibition of protein synthesis and cell death (A. D. O'Brien *et al.*. "Shiga and Shiga-like toxins, Microbial Rev., 51: 206-220 [1987]).

The role of verotoxins in the pathogenesis of *E. coli* O157:H7 infections has been further studied in animal models. Infection or toxin challenge of laboratory animals do not produce all the pathologies and symptoms of hemorrhagic colitis. HUS, and TTP which occur in humans. Glomerular damage is noticeably absent. Nonetheless, experiments using animal models implicate verotoxins as the direct cause of hemorrhagic colitis, microvascular damage leading to the failure of kidneys and other organs and CNS neuropathies.

For example, Barrett. et al. delivered VT2 into the peritoneal cavity of rabbits using mini-osmotic pumps (J. J. Barrett et al., "Continuous peritoneal infusion of shiga-like toxin II (SLTII) as a model for SLT II-induced diseases," J. Infect. Dis., 159: 774-777 [1989]). In three days, most animals receiving the toxin developed diarrhea, with intestinal lesions resembling those seen in humans with hemorrhagic colitis. Although there was some evidence of renal dysfunction, none of the rabbits developed HUS. Beery, et al. showed that VT2, when administered intraperitoneally or intravenously to adult mice, produces lesions of the kidneys and colon (J. T. Beery et al., "Cytotoxic activity of Escherichia coli O157:H7 culture filtrate on the mouse colon and kidney," Curr. Microbiol., 11: 335-342 [1984]). Histologic lesions in the kidney included accumulation of numerous exfoliated collecting tubules and marked intracellular vacuolation of proximal convoluted tubular cells.

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Sjögren et. al. studied the pathogenesis of an entero-adherent strain of E. coli (RDEC-1) lysogenized with a VT1-containing bacteriophage (VT1-producing RDEC-1) (R. Sjögren et al., "Role of Shiga-like toxin I in bacterial enteritis: comparison between isogenic Escherichia coli strains induced in rabbits." Gastroenterol., 106: 306-317 [1994]). In this study, rabbits were challenged with RDEC-1 or VT1-producing RDEC-1 and studied for onset of disease. The VT1-producing variant induced a severe, non-invasive, entero-adherent infection in rabbits which was characterized by serious histological lesions with vascular changes, edema and severe epithelial inflammation. Importantly, vascular changes consistent with endothelial damage were seen in infected animals that was similar to intestinal microvascular changes in humans with E. coli O157:H7 infection. Based on these observations, they concluded that VT1 is an important virulence factor in enterohemorrhagic E. coli O157:H7 infection.

Fuji et. al. described a model in which mice were treated for three days with streptomycin followed by a simultaneous challenge of E. coli O157:H7 orally, and mitomycin intraperitoneally (J. Fuji et al., "Direct evidence of neuron impairment by oral infection with Verotoxin-producing Escherichia coli O157:H7 in mitomycin-treated mice." Infect. Immun.. 62: 3447-34453 [1994]). All of the animals died within four days. Immunoelectron-microscopy strongly suggested that death was due to the toxic effects of VT2v (a structural variant of VT2), on both the endothelial cells and neurons in the central nervous system which resulted in fatal acute encephalopathy.

Wadolkowski et al. studied colonization of E. coli O157:H7 in mice. Mice were treated with streptomycin and fed 10¹⁰ E. coli O157:H7 (E. A. Wadolkowski et al., "Mouse

model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7." Infect. Immun., 58: 2438-2445 [1990]; and E. A. Wadolkowski *et al.*, "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shigalike toxin Type II." Infect. Immun., 58: 3959-3965 [1990]). All of the mice died due to severe, disseminated, acute necrosis of proximal convoluted tubules. In mouse models, glomerular damage was not observed, but toxic acute renal tubular necrosis was observed which is characteristic of some HUS patients. The failure of mice to show glomerular damage is thought to be due to the absence of a functional globotriaosyl ceramide receptor specific for verotoxins in the glomeruli of the kidneys. Administration of VT2 subunit-specific monoclonal antibodies prior to infection prevented all pathology and death.

E. Current Therapeutic Approaches

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EDMIN -MID DESCRIPTION

E. coli O157:H7 disease is not adequately controlled by current therapy. Patient treatment is tailored to manage fluid and electrolyte disturbances, anemia, renal failure and hypertension. Although E. coli O157:H7 is susceptible to common antibiotics, the role of antibiotics in the treatment of infection has questionable merit. In both retrospective and prospective studies, prophylaxis or treatment with antibiotics such as trimethoprimsulfamethoxazole, there was either no benefit or an increased risk of developing HUS (T. N. Bokete et al., "Shiga-like toxin producing Escherichia coli in Seattle children: a prospective study," Gastroenterol., 105: 1724-1731 [1993]; A. T. Pavia et al., "Hemolytic uremicsyndrome during an outbreak of Escherichia coli O157:H7 infections in institutions for mentally retarded persons: clinical and epidemiologic observations." J. Pedatr., 116: 544-551 [1990]; F. Proulx et al., "Randomized, controlled trial of antibiotic therapy for Escherichia coli O157:H7 enteritis." J. Pediatr., 121: 299-303 [1992]; and A. L. Carter et al., "A severe outbreak of Escherichia coli O157:H7-associated hemorrhagic colitis in a nursing home." New Eng. J. Med., 317: 1496-1500 [1987]).

The mechanisms by which antibiotics increase the risk of infection or related complications might involve enhancement of toxin production, release of toxins from killed organisms, or alteration of normal competing intestinal flora allowing for pathogen overgrowth (M. A. Karmali, "Infection by Verocytotoxin-producing Escherichia coli," Clin. Microbiol. Rev., 2: 15-38 [1989]). A further concern in the use of antibiotics is the potential acquisition of antimicrobial resistance by E. coli O157:H7 (C. R. Dorn, "Review of foodborne outbreak of Escherichia coli O157:H7 infection in the western United States," JAVMA 203: 1583-1587 [1993]).

In addition, by the time symptoms are serious enough to attract medical attention, it is likely that verotoxins are already entering the systemic circulation or will do so shortly thereafter. Although antimicrobials may help to prevent pathology resulting from the action of toxin on the bowel lumen. However, by the time symptoms of HUS have developed, the patient has ceased shedding organisms. Thus, antimicrobial treatment during HUS disease is of less value, and often contraindicated, due to the increased risk of complications associated with administration of antimicrobials to patients susceptible to development of HUS. Importantly, there is currently no antitoxin commercially available for use in treating affected patients. What is needed is a means to block the progression of disease, without the complications associated with antimicrobial treatment.

DESCRIPTION OF THE DRAWINGS

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Figure 1 is an SDS-PAGE of rVT1 and rVT2.

Figure 2 shows HPLC results for rVT1 and rVT2.

Figure 3 shows rVT1 and rVT2 toxicity in Vero cell culture.

Figure 4 shows EIA reactivity of rVT1 and rVT2 antibodies to rVT1.

Figure 5 shows EIA reactivity of rVT1 and rVT2 Antibodies to rVT2.

Figure 6 shows Western Blot reactivity of rVT1 and rVT2 antibodies to rVT's:

Panel 6A contains preimmune lgY:

Panel 6B contains rVT1 IgY; and

Panel 6C contains rVT2 IgY.

Figure 7 shows neutralization of rVT1 cytotoxicity in Vero cells.

Figure 8 shows neutralization of rVT2 cytotoxicity in Vero cells.

Figure 9 shows renal sections from E. coli O157:H7-infected mice treated with lgY

Panel 9A shows a representative kidney section from a mouse treated with preimmune IgY;

Panel 9B shows a representative kidney sections from a mouse treated with rVT1; and

Panel 9C shows a representative kidney section from a mouse treated with rVT2 IgY.

Figure 10 shows the fusion constructs of VT components and affinity tags.

DEFINITIONS

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To facilitate understanding of the invention, a number of terms are defined below.

As used herein, the term "neutralizing" is used in reference to antitoxins, particularly antitoxins comprising antibodies, which have the ability to prevent the pathological actions of the toxin against which the antitoxin is directed.

As used herein, the term "overproducing" is used in reference to the production of toxin polypeptides in a host cell, and indicates that the host cell is producing more of the toxin by virtue of the introduction of nucleic acid sequences encoding the toxin polypeptide than would be expressed by the host cell absent the introduction of these nucleic acid sequences. To allow ease of purification of toxin polypeptides produced in a host cell it is preferred that the host cell express or overproduce the toxin polypeptide at a level greater than 1 mg/liter of host cell culture.

As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (i.e., an E. coli verotoxin and/or fragments thereof) joined to an exogenous protein fragment (the fusion partner which consists of a non-toxin protein). The fusion partner may enhance solubility of the E. coli protein as expressed in a host cell, may provide an "affinity tag" to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both. If desired, the fusion protein may be removed from the protein of interest (i.e., toxin protein or fragments thereof) prior to immunization by a variety of enzymatic or chemical means known to the art.

As used herein, the term "affinity tag" refers to such structures as a "poly-histidine tract" or "poly-histidine tag," or any other structure or compound which facilitates the purification of a recombinant fusion protein from a host cell, host cell culture supernatant, or both. As used herein, the term "flag tag" refers to short polypeptide marker sequence useful for recombinant protein identification and purification.

As used herein, the terms "poly-histidine tract" and "poly-histidine tag," when used in reference to a fusion protein refers to the presence of two to ten histidine residues at either the amino- or carboxy-terminus of a protein of interest. A poly-histidine tract of six to ten residues is preferred. The poly-histidine tract is also defined functionally as being a number of consecutive histidine residues added to the protein of interest which allows the affinity purification of the resulting fusion protein on a nickel-chelate column.

As used herein, the term "chimeric protein" refers to two or more coding sequences obtained from different genes, that have been cloned together and that, after translation, act as

a single polypeptide sequence. Chimeric proteins are also referred to as "hybrid proteins." As used herein, the term "chimeric protein" refers to coding sequences that are obtained from different species of organisms, as well as coding sequences that are obtained from the same species of organisms.

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As used herein, the term "protein of interest" refers to the protein whose expression is desired within the fusion protein. In a fusion protein, the protein of interest will be joined or fused with another protein or protein domain, the fusion partner, to allow for enhanced stability of the protein of interest and/or ease of purification of the fusion protein.

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As used herein, the term "maltose binding protein" and "MBP" refers to the maltose binding protein of *E. coli*. A portion of the maltose binding protein may be added to a protein of interest to generate a fusion protein: a portion of the maltose binding protein may merely enhance the solubility of the resulting fusion protein when expressed in a bacterial host. On the other hand, a portion of the maltose binding protein may allow affinity purification of the fusion protein on an amylose resin.

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As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, antitoxins are purified by removal of contaminating non-immunoglobulin proteins: they are also purified by the removal of substantially all immunoglobulin that does not bind toxin. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind toxin results in an increase in the percent of toxin-reactive immunoglobulins in the sample. In another example, recombinant toxin polypeptides are expressed in bacterial host cells and the toxin polypeptides are purified by the removal of host cell proteins: the percent of recombinant toxin polypeptides is thereby increased in the sample.

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The term "recombinant DNA molecule" as used herein refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed from a recombinant DNA molecule.

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The term "native protein" as used herein refers to a protein which is isolated from a natural source as opposed to the production of a protein by recombinant means.

As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

As used herein "soluble" when in reference to a protein produced by recombinant DNA technology in a host cell, is a protein which exists in solution in the cytoplasm of the host cell: if the protein contains a signal sequence, the soluble protein is exported to the periplasmic space in bacterial hosts and is secreted into the culture medium of eukaryotic cells capable of secretion or by bacterial hosts possessing the appropriate genes. In contrast, an insoluble protein is one which exists in denatured form inside cytoplasmic granules (called an inclusion bodies) in the host cell. High level expression (i.e., greater than 1 mg recombinant protein/liter of bacterial culture) of recombinant proteins often results in the expressed protein being found in inclusion bodies in the bacterial host cells. A soluble protein is a protein which is not found in an inclusion body inside the host cell or is found both in the cytoplasm and in inclusion bodies and in this case the protein may be present at high or low levels in the cytoplasm.

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A distinction is drawn between a soluble protein (i.e., a protein which when expressed in a host cell is produced in a soluble form) and a "solubilized" protein. An insoluble recombinant protein found inside an inclusion body may be solubilized (i.e., rendered into a soluble form) by treating purified inclusion bodies with denaturants such as guanidine hydrochloride, urea or sodium dodecyl sulfate (SDS). These denaturants must then be removed from the solubilized protein preparation to allow the recovered protein to renature (refold). Not all proteins will refold into an active conformation after solubilization in a denaturant and removal of the denaturant. Many proteins precipitate upon removal of the denaturant. SDS may be used to solubilize inclusion bodies and will maintain the proteins in solution at low concentration. However, dialysis will not always remove all of the SDS (SDS can form micelles which do not dialyze out): therefore, SDS-solubilized inclusion body protein is soluble but not refolded.

As used herein, the term "reporter reagent" or "reporter molecule" is used in reference to compounds which are capable of detecting the presence of antibody bound to antigen. For example, a reporter reagent may be a colorimetric substance, which is attached to an enzymatic substrate. Upon binding of antibody and antigen, the enzyme acts on its substrate and causes the production of a color. Other reporter reagents include, but are not limited to fluorogenic and radioactive compounds or molecules.

As used herein the term "signal" is used in reference to the production of a sign that a reaction has occurred, for example, binding of antibody to antigen. It is contemplated that signals in the form of radioactivity, fluorogenic reactions, and enzymatic reactions will be

used with the present invention. The signal may be assessed quantitatively as well as qualitatively.

As used herein, the term "therapeutic amount" refers to that amount of antitoxin required to neutralize the pathologic effects of *E. coli* toxin in a subject.

As used herein, the term "acute intoxication" is used in reference to cases of E, coli infection in which the patient is currently suffering from the effects of toxin (e.g., E, coli verotoxins or enterotoxins). Signs and symptoms of intoxication with the toxin may be immediately apparent. Or, the determination of intoxication may require additional testing, such as detection of toxin present in the patient's fecal material.

As used herein, the term "at risk" is used in references to individuals who have been exposed to *E. coli* and may suffer the symptoms associated with infection or disease with these organisms, especially due to the effects of verotoxins.

SUMMARY OF THE INVENTION

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The present invention relates to antitoxin therapy for humans and other animals. Antitoxins which neutralize the pathologic effects of *E. coli* toxins are generated by immunization of avian hosts with recombinant toxin fragments. In one embodiment, the present invention contemplates a method of treatment administering at least one antitoxin directed against at least a portion of an *Escherichia coli* verotoxin in an aqueous solution in therapeutic amount that is administrable to an intoxicated subject. It is contemplated that the intoxicated subject will be either an adult or a child.

In a preferred embodiment, the *E. coli* verotoxin is recombinant. In one embodiment, the antitoxin is an avian antitoxin. In an alternative embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence. In one embodiment of the *E. coli* fusion protein, the fusion protein comprises a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT2 sequence.

Various routes of administration, are contemplated for providing the *E. coli* antitoxin(s) to an affected individual, including but not limited to, parenteral as well as oral routes of administration. In a particularly preferred embodiment, the route of administration is parenteral.

The present invention also includes the embodiment of a method of prophylactic treatment in which an antitoxin directed against at least one *E. coli* verotoxin in an aqueous

solution in therapeutic amount that is parenterally administrable, and is administered to at least one subject at risk of diarrheal disease. It one embodiment, the antitoxin is parenterally administered.

In one embodiment, the subject is at risk of developing extra-intestinal complications of *E. coli* infections, including but not limited to, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, etc.

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The present invention also includes the embodiment of a composition which comprises neutralizing antitoxin directed against at least one *E. coli* verotoxin in an aqueous solution in therapeutic amounts. In one particularly preferred embodiment, the *E. coli* verotoxin is a recombinant toxin. In an alternative embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *E. coli* verotoxin VT1 sequence. In another embodiment, the recombinant *E. coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *E. coli* verotoxin VT2 sequence. In yet another embodiment, the composition of the antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin. In one embodiment, the portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT1. In an alternative embodiment, the portion of *Escherichia coli* is selected from the group consisting of subunit A and subunit B of VT2. Indeed, the invention contemplates an antitoxin that is directed against a portion of at least one *Escherichia coli* verotoxin. In one embodiment, the antitoxin is an avian antitoxin.

The present invention also comprises a method of treatment of enteric bacterial infections comprising administering an avian antitoxin directed against at least one verotoxin produced by *E. coli* in an aqueous solution in therapeutic amount, to at least one infected subject. In one preferred embodiment, the avian antitoxin is administered parenterally.

In another embodiment, the *E. coli* is selected from the group consisting of *Escherichia coli* serotypes O157:H7, O1:NM: O2:H5: O2:H7: O4:NM: O4:H10: O5:NM: O5:H16: O6:H1: O18:NM: O18:H7: O25:NM: O26:NM: O26:H11: O26:H32: O38:H21: O39:H4: O45:H2: O50:H7: O55:H7; O55:H10: O82:H8: O84:H2: O91:NM: O91:H21: O103:H2: O111:NM: O111:H8: O111:H30: O111:H34: O113:H7: O113:H21: O114:H48; O115:H10: O117:H4: O118:H12: O118:H30: O121:NM: O121:H19: O125:NM; O125:H8: O126:NM: O126:H8: O128:NM: O128:H2: O128:H8: O128:H12: O128:H25: O145:NM; O125:H25: O146:H21: O153:H25: O157:NM: O163:H19: O165:NM: O165:19: and O165:H25. In one embodiment, the antitoxin comprises antitoxin directed against at least one

Escherichia coli verotoxin. In another embodiment, the antitoxin is cross-reacts with at least one Escherichia coli verotoxin. In yet another embodiment, the antitoxin is reactive against toxins produced by members of the genus Shigella, including S. dysenteriae.

The present invention also contemplates uses for the toxin fragments in vaccines and diagnostic assays. The fragments may be used separately as purified, soluble antigens or, alternatively, in mixtures or "cocktails." The present invention thus comprises a method for detecting Escherichia coli verotoxin in a sample in which a sample an antitoxin raised against Escherichia coli verotoxin, and a reporter reagent capable of binding the antitoxin are provided. The antitoxin is added to the sample, so that the antitoxin binds to the E. coli verotoxin in the sample. In one embodiment, the antitoxin is an avian antitoxin. In an alternative embodiment, the method further comprises the steps of washing unbound antitoxin from the sample, adding at least one reporter reagent to the sample, so that said reporter reagent binds to any antitoxin that is bound, washing the unbound reporter reagent from the sample and detecting the reporter reagent bound to the antitoxin bound to the Escherichia coli verotoxin, so that the verotoxin is detected. In one embodiment, the detecting is accomplished through any means, such as enzyme immunoassay, radioimmunoassay, fluorescence immunoassay, flocculation, particle agglutination, and in situ chromogenic assay. In one preferred embodiment, the sample is a biological sample. In an alternative preferred embodiment, the sample is an environmental sample.

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DESCRIPTION OF THE INVENTION

The present invention contemplates treating humans and other animals intoxicated with at least one bacterial toxin. It is contemplated that administration of antitoxin will be used to treat patients effected by or at risk of symptoms due to the action of bacterial toxins. It is also contemplated that the antitoxin will be used in a diagnostic assay to detect the presence of toxins in samples. The organisms, toxins and individual steps of the present invention are described separately below.

I. Antibodies Directed Against E. coli and Associated Toxins

A preferred embodiment of the method of the present invention is directed toward obtaining antibodies against various *E. coli* serotypes, their toxins, enzymes or other metabolic by-products, cell wall components, or synthetic or recombinant versions of any of these compounds. It is contemplated that these antibodies will be produced by immunization

of humans or other animals. It is not intended that the present invention be limited to any particular toxin or any species of organism. In one embodiment, toxins from all *E. coli* serotypes are contemplated as immunogens. Examples of these toxins include the verotoxins VT1 and VT2.

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It is not intended that antibodies produced against one toxin will only be used against that toxin. It is contemplated that antibodies directed against one toxin may be used as an effective therapeutic against one or more toxin(s) produced by other *E. coli* serotypes, or other toxin producing organisms (e.g., Shigella, Bacillus cereus, Staphylococcus aureus, Streptococcus mutans, Acinetobacter calcoaceticus, Pseudomonas aeruginosa, other Pseudomonas species, Vibrio species, Clostridium species, etc.). It is further contemplated that antibodies directed against the portion of the toxin which binds to mammalian membranes can also be used against other organisms. It is contemplated that these membrane binding domains are produced synthetically and used as immunogens.

II. Obtaining Antibodies In Non-Mammals

A preferred embodiment of the method of the present invention for obtaining antibodies involves immunization. However, it is also contemplated that antibodies may be obtained from non-mammals without immunization. In the case where no immunization is contemplated, the present invention may use non-mammals with preexisting antibodies to toxins as well as non-mammals that have antibodies to whole organisms by virtue of reactions with the administered antigen. An example of the latter involves immunization with synthetic peptides or recombinant proteins sharing epitopes with whole organism components.

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In a preferred embodiment, the method of the present invention contemplates immunizing non-mammals with bacterial toxin(s). It is not intended that the present invention be limited to any particular toxin. In one embodiment, toxins from all *E. coli* serotypes are contemplated as immunogens.

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A particularly preferred embodiment involves the use of bacterial toxin protein or fragments of toxin proteins produced by molecular biological means (i.e., recombinant toxin proteins). In a preferred embodiment, the immunogen comprises recombinant VT1 and/or VT2.

When immunization is used, the preferred non-mammal is from the class Aves. All birds are contemplated (e.g., duck, ostrich, emu, turkey, etc.). A preferred bird is a chicken. Importantly, chicken antibody does not fix mammalian complement (See H.N. Benson et al.,

J. Immunol. 87:616 [1961]). Thus, chicken antibody will normally not cause a complement-dependent reaction (A.A. Benedict and K. Yamaga. "Immunoglobulins and Antibody Production in Avian Species." in Comparative Immunology (J.J. Marchaloni, ed.), pp. 335-375. Blackwell. Oxford [1966]). Thus, the preferred antitoxins of the present invention will not exhibit complement-related side effects observed with antitoxins presently known.

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When birds are used, it is contemplated that the antibody will be obtained from either the bird serum or the egg. A preferred embodiment involves collection of the antibody from the egg. Laying hens transport immunoglobulin to the egg yolk ("IgY") in concentrations equal to or exceeding that found in serum (See R. Patterson et al., J. Immunol. 89:272 (1962); and S.B. Carroll and B.D. Stollar, J. Biol. Chem. 258:24 [1983]). In addition, the large volume of egg yolk produced vastly exceeds the volume of serum that can be safely obtained from the bird over any given time period. Finally, the antibody from eggs is more pure and more homogeneous: there is far less non-immunoglobulin protein (as compared to serum) and only one class of immunoglobulin is transported to the yolk.

When considering immunization with toxins, one may consider modification of the toxins to reduce the toxicity. In this regard, it is not intended that the present invention be limited by immunization with modified toxin. Unmodified ("native") toxin is also contemplated as an immunogen.

It is also not intended that the present invention be limited by the type of modification -- if modification is used. The present invention contemplates all types of toxin modification, including chemical and heat treatment of the toxin. In one embodiment, glutaraldehyde treatment of the toxin is contemplated. In an alternative embodiment, formaldehyde treatment of the toxin is contemplated.

It is not intended that the present invention be limited to a particular mode of immunization: the present invention contemplates all modes of immunization, including subcutaneous, intramuscular, intraperitoneal, and intravenous or intravascular injection, as well as *per os* administration of immunogen.

The present invention further contemplates immunization with or without adjuvant. As used herein, the term "adjuvant" is defined as a substance known to increase the immune response to other antigens when administered with other antigens. If adjuvant is used, it is not intended that the present invention be limited to any particular type of adjuvant -- or that the same adjuvant, once used, be used all the time. While the present invention contemplates all types of adjuvant, whether used separately or in combinations, the preferred use of

adjuvant is the use of Complete Freund's Adjuvant followed sometime later with Incomplete Freund's Adjuvant. The invention also contemplates the use of fowl adjuvant commercially available from RIBI, as well as Quil A adjuvant commercially available from Accurate Chemical and Scientific Corporation, and Gerbu adjuvant also commercially available (GmDP: C.C. Biotech Corp.).

When immunization is used, the present invention contemplates a wide variety of immunization schedules. In one embodiment, a chicken is administered toxin(s) on day zero and subsequently receives toxin(s) in intervals thereafter. It is not intended that the present invention be limited by the particular intervals or doses. Similarly, it is not intended that the present invention be limited to any particular schedule for collecting antibody. The preferred collection time is sometime after day 35.

Where birds are used and collection of antibody is performed by collecting eggs, the eggs may be stored prior to processing for antibody. It is preferred that eggs be stored at 4°C for less than one year.

It is contemplated that chicken antibody produced in this manner can be bufferextracted and used analytically. While unpurified, this preparation can serve as a reference for activity of the antibody prior to further manipulations (e.g., immunoaffinity purification).

III. Increasing The Effectiveness Of Antibodies

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When purification is used, the present invention contemplates purifying to increase the effectiveness of both non-mammalian antitoxins and mammalian antitoxins. Specifically, the present invention contemplates increasing the percent of toxin-reactive immunoglobulin. The preferred purification approach for avian antibody is polyethylene glycol (PEG) separation.

The present invention contemplates that avian antibody be initially purified using simple, inexpensive procedures. In one embodiment, chicken antibody from eggs is purified by extraction and precipitation with PEG. PEG purification exploits the differential solubility of lipids (which are abundant in egg yolks) and yolk proteins in high concentrations of PEG 8000 (Polson et al., Immunol. Comm. 9:495 [1980]). The technique is rapid, simple, and relatively inexpensive and yields an immunoglobulin fraction that is significantly more pure, in terms of contaminating non-immunoglobulin proteins than the comparable ammonium sulfate fractions of mammalian sera and horse antibodies. The majority of the PEG is removed from the precipitated chicken immunoglobulin by treatment with ethanol. Indeed,

PEG-purified antibody is sufficiently pure that the present invention contemplates the use of PEG-purified antitoxins in the passive immunization of intoxicated humans and animals.

IV. Treatment

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The present invention contemplates antitoxin therapy for humans and other animals intoxicated by bacterial toxins. A preferred method of treatment is by parenteral administration of antitoxin.

A. Dosage Of Antitoxin

It was noted by way of background that a balance must be struck when administering currently available antitoxin which is usually produced in large animals such as horses: sufficient antitoxin must be administered to neutralize the toxin, but not so much antitoxin as to increase the risk of untoward side effects. These side effects are caused by: i) patient sensitivity to foreign (e.g. horse) proteins; ii) anaphylactic or immunogenic properties of non-immunoglobulin proteins; iii) the complement fixing properties of mammalian antibodies: and/or iv) the overall burden of foreign protein administered. It is extremely difficult to strike this balance when, as noted above, the degree of intoxication (and hence the level of antitoxin therapy needed) can only be approximated.

The present invention contemplates significantly reducing side effects so that this balance is more easily achieved. Treatment according to the present invention contemplates reducing side effects by using PEG-purified antitoxin from birds.

In one embodiment, the treatment of the present invention contemplates the use of PEG-purified antitoxin from birds. The use of yolk-derived, PEG-purified antibody as antitoxin allows for the administration of: 1) non (mammalian)-complement-fixing, avian antibody; 2) a less heterogeneous mixture of non-immunoglobulin proteins: and 3) less total protein to deliver the equivalent weight of active antibody present in currently available antitoxins. The non-mammalian source of the antitoxin makes it useful for treating patients who are sensitive to horse or other mammalian sera.

As is true in cases of botulism, the degree of an individual's exposure to *E. coli* toxin and the prognosis are often difficult to assess, and depend upon a number of factors (*e.g.*, the quantity of contaminated food ingested, the toxigenicity and serotype of *E. coli* strain ingested, etc.). Thus, the clinical presentation of a patient is usually a more important consideration than a quantitative diagnostic test, for determination of dosage in antitoxin

administration. Indeed, for many toxin-associated diseases (e.g., botulism, tetanus, diphtheria, etc.), there is no rapid, quantitative test to detect the presence of the toxin or organism. Rather, these toxin-associated diseases are medical emergencies which mandate immediate treatment. Confirmation of the etiologic agent must not delay the institution of therapy, as the condition of an affected patient may rapidly deteriorate. In addition to the initial treatment with antitoxin, subsequent doses may be indicated, as the patient's disease progresses. The dosage and timing of these subsequent doses is dependent upon the signs and symptoms of disease in each individual patient.

It is contemplated that the administration of antitoxin to an affected individual would involve an initial injection of an approximately 10 ml dose of immune globulin (with less than approximately 1 gram of total protein). In one preferred embodiment, it is contemplated that at least 50% of the initial injection comprises immune globulin. It is also contemplated that more purified immune globulin be used for treatment, wherein approximately 90% of the initial injection comprises immune globulin. When more purified immune globulin is used, it is contemplated that the total protein will be less than approximately 100 milligrams. It is also contemplated that additional doses be given, depending upon the signs and symptoms associated with *E. coli* verotoxin disease progression.

B. Delivery Of Antitoxin

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Although it is not intended to limit the route of delivery, the present invention contemplates a method for antitoxin treatment of bacterial intoxication in which delivery of antitoxin is parenteral or oral.

In one embodiment, antitoxin is parenterally administered to a subject in an aqueous solution. It is not intended that the parenteral administration be limited to a particular route. Indeed, it is contemplated that all routes of parenteral administration will be used. In one embodiment, parenteral administration is accomplished via intramuscular injection. In an alternative embodiment, parenteral administration is accomplished via intravenous injection.

In another embodiment, antitoxin is delivered in a solid form (e.g., tablets). In an alternative embodiment antitoxin is delivered in an aqueous solution. When an aqueous solution is used, the solution has sufficient ionic strength to solubilize antibody protein, yet is made palatable for oral administration. The delivery solution may also be buffered (e.g., carbonate buffer, pH 9.5) which can neutralize stomach acids and stabilize the antibodies when the antibodies are administered orally. In one embodiment the delivery solution is an

aqueous solution. In another embodiment the delivery solution is a nutritional formula. Preferably, the delivery solution is infant or a dietary supplement formula (e.g., Similac®). Ensure®, and Enfamil®). Yet another embodiment contemplates the delivery of lyophilized antibody encapsulated or microencapsulated inside acid-resistant compounds.

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Methods of applying enteric coatings to pharmaceutical compounds are well known to the art (companies specializing in the coating of pharmaceutical compounds are available: for example. The Coating Place [Verona. WI] and AAI [Wilmington. NC]). Enteric coatings which are resistant to gastric fluid and whose release (i.e., dissolution of the coating to release the pharmaceutical compound) is pH dependent are commercially available (for example, the polymethacrylates Eudragit® L and Eudragit® S [Röhm Tech Inc., Malden, MA]). Eudragit® S is soluble in intestinal fluid from pH 7.0; this coating can be used to microencapsulate lyophilized antitoxin antibodies and the particles are suspended in a solution having a pH above or below pH 7.0 for oral administration. The microparticles will remain intact and undissolved until they reached the intestines where the intestinal pH would cause them to dissolve thereby releasing the antitoxin.

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The invention contemplates a method of treatment which can be administered for treatment of acute intoxication. In one embodiment, antitoxin is administered orally in either a delivery solution or in tablet form, in therapeutic dosage, to a subject intoxicated by the bacterial toxin which served as immunogen for the antitoxin. In another embodiment of treatment of acute intoxication, a therapeutic dosage of the antitoxin in a delivery solution, is parenterally administered.

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The invention also contemplates a method of treatment which can be administered prophylactically. In one embodiment, antitoxin is administered orally, in a delivery solution, in therapeutic dosage, to a subject, to prevent intoxication of the subject by the bacterial toxin which served as immunogen for the production of antitoxin. In another embodiment, antitoxin is administered orally in solid form such as tablets or as microencapsulated particles. Microencapsulation of lyophilized antibody using compounds such as Eudragit® (Rohm GmbH) or polyethylene glycol, which dissolve at a wide range of pH units, allows the oral administration of solid antitoxin in a liquid form (i.e., a suspension) to recipients unable to tolerate administration of tablets (e.g., children or patients on feeding tubes). In one preferred embodiment the subject is a child. In another embodiment, antibody raised against whole bacterial organism is administered orally to a subject, in a delivery solution, in therapeutic

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dosage. In yet another preferred embodiment of prophylactic treatment, a therapeutic dosage of the antitoxin in a delivery solution, is parenterally administered.

V. Multivalent Vaccines Against E. coli Strains

The invention contemplates the generation of multivalent vaccines for the protection of an organism (particularly humans) against several *E. coli* strains. Of particular interest is a vaccine which stimulates the production of a humoral immune response to *E. coli* O157:H7, O26:H11. O113:H21. O91:H21, and O111:NM, in humans. The antigens comprising the vaccine preparation may be native or recombinantly produced toxin proteins from the *E. coli* serotypes listed above. When native toxin proteins are used as immunogens they are generally modified to reduce the toxicity. It is contemplated that glutaraldehyde-modified toxin proteins will be used. In an alternative embodiment, is formaldehyde-modified toxin proteins will be used.

The invention contemplates that recombinant E, coli verotoxin proteins be used in conjunction with either native toxins or toxoids from other organisms as antigens in a multivalent vaccine preparation. It is also contemplated that recombinant E coli toxin proteins be used in the multivalent vaccine preparation.

Vl. Detection Of Toxin

The invention contemplates detecting bacterial toxin in a sample. The term "sample" in the present specification and claims is used in its broadest sense. On the one hand it is meant to include a specimen or culture (e.g., microbiological cultures). On the other hand, it is meant to include both biological and environmental samples.

Biological samples may be animal, including human, fluid, solid (e.g., stool) or tissue, as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may be obtained from all of the various families of common domestic animals, including but not limited, to bovines (e.g., cattle), ovines (e.g., sheep), caprines (e.g., goats), porcines (e.g., swine), equines (e.g., horses), canines (e.g., dogs), lagamorphs (e.g., rabbits), and felines (e.g., cats), etc. It is also intended that samples may be obtained from feral or wild animals, including, but not limited to, such animals as ungulates (e.g., deer), bear, fish, lagamorphs, rodents, etc.

Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing

instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

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The invention contemplates detecting bacterial toxin by a competitive immunoassay method that utilizes recombinant toxin VT1 and toxin VT2 proteins. antibodies raised against recombinant bacterial toxin proteins. A fixed amount of the recombinant toxin proteins are immobilized to a solid support (e.g., a microtiter plate) followed by the addition of a biological sample suspected of containing a bacterial toxin. The biological sample is first mixed with affinity-purified or PEG fractionated antibodies directed against the recombinant toxin protein. A reporter reagent is then added which is capable of detecting the presence of antibody bound to the immobilized toxin protein. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. If toxin is present in the sample, this toxin will compete with the immobilized recombinant toxin protein for binding to the anti-recombinant antibody thereby reducing the signal obtained following the addition of the reporter reagent. A control is employed where the antibody is not mixed with the sample. This gives the highest (or reference) signal.

The invention also contemplates detecting bacterial toxin by a "sandwich" immunoassay method that utilizes antibodies directed against recombinant bacterial toxin proteins. Affinity-purified antibodies directed against recombinant bacterial toxin proteins are immobilized to a solid support (e.g., microtiter plates). Biological samples suspected of containing bacterial toxins are then added followed by a washing step to remove substantially all unbound antitoxin. The biological sample is next exposed to the reporter substance, which binds to antitoxin and is then washed free of substantially all unbound reporter substance. The reporter substance may comprise an antibody with binding specificity for the antitoxin attached to a molecule which is used to identify the presence of the reporter substance. Identification of the reporter substance in the biological tissue indicates the presence of the bacterial toxin.

It is also contemplated that bacterial toxin be detected by pouring liquids (e.g., soups and other fluid foods and feeds including nutritional supplements for humans and other animals) over immobilized antibody which is directed against the bacterial toxin. It is contemplated that the immobilized antibody will be present in or on such supports as cartridges, columns, beads, or any other solid support medium. In one embodiment, following

the exposure of the liquid to the immobilized antibody, unbound toxin is substantially removed by washing. The liquid is then exposed to a reporter substance which detects the presence of bound toxin. In a preferred embodiment the reporter substance is an enzyme, fluorescent dye, or radioactive compound attached to an antibody which is directed against the toxin (i.e., in a "sandwich" immunoassay). It is also contemplated that the detection system will be developed as necessary (e.g., the addition of enzyme substrate in enzyme systems: observation using fluorescent light for fluorescent dye systems: and quantitation of radioactivity for radioactive systems).

10 EXPERIMENTAL

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The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and arc not to be construed as limiting the scope thereof.

In the disclosure which follows, the following abbreviations apply: °C (degrees Centigrade): rpm (revolutions per minute): BSA (bovine serum albumin): ELISA (enzymelinked immunosorbent assay): IgG (immunoglobulin G): IgY (immunoglobulin Y): IP (intraperitoneal): SC (subcutaneous): H₂O (water): HCl (hydrochloric acid): LD₁₀₀ (lethal dose for 100% of experimental animals); aa (amino acid); HPLC (high performance liquid chromatography): Kda (kilodaltons): gm (grams): μg (micrograms): mg (milligrams): ng (nanograms): µl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm (micrometer): M (molar): mM (millimolar): MW (molecular weight): sec (seconds): min(s) (minute/minutes): hr(s) (hour/hours): MgCl₂ (magnesium chloride): NaCl (sodium chloride): Na₂CO₃ (sodium carbonate): OD₂₈₀ (optical density at 280 nm): OD₆₀₀ (optical density at 600 nm); PAGE (polyacrylamide gel electrophoresis); PBS [phosphate buffered saline (150 mM NaCl. 10 mM sodium phosphate buffer, pH 7.2)]; PEG (polyethylene glycol); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); w/v (weight to volume); v/v (volume to volume): Amicon (Amicon, Inc., Beverly, MA): Amresco (Amresco, Inc., Solon, OH); ATCC (American Type Culture Collection, Rockville, MD); BBL (Baltimore Biologies Laboratory. (a division of Becton Dickinson), Cockeysville, MD); Becton Dickinson (Becton Dickinson Labware. Lincoln Park. NJ); BioRad (BioRad. Richmond. CA): Biotech (C-C Biotech Corp., Poway, CA): Charles River (Charles River Laboratories, Wilmington, MA); Falcon (e.g. Baxter Healthcare Corp., McGaw Park, IL and Becton Dickinson): Fisher Biotech (Fisher Biotech, Springfield, NJ); GIBCO (Grand Island Biologic Company/BRL, Grand Island, NY): Mallinckrodt (a division of Baxter Healthcare Corp., McGaw Park, IL);

Millipore (Millipore Corp., Marlborough, MA); New England Biolabs (New England Biolabs, Inc., Beverly, MA); Novagen (Novagen, Inc., Madison, WI): Pharmacia (Pharmacia, Inc., Piscataway, NJ): Qiagen (Qiagen, Chatsworth, CA); Showdex (Showa Denko America, Inc., New York, NY): Sigma (Sigma Chemical Co., St. Louis, MO): RIBI (RIBI Immunochemical Research Inc., Hamilton, MT); Accurate Chemical and Scientific Corp. (Accurate Chemical and Scientific Corp., Hicksville, NY); Kodak (Eastman-Kodak, Rochester, NY): and Stratagene (Stratagene, La Jolla, CA).

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When a recombinant protein is described in the specification it is referred to in a short-hand manner by the amino acids in the toxin sequence present in the recombinant protein rounded to the nearest 10. The specification gives detailed construction details for all recombinant proteins such that one skilled in the art will know precisely which amino acids are present in a given recombinant protein.

The first set of Examples (Examples 1-5) was designed to develop an antidote to *E. coli* O157:H7 verotoxins and evaluate its effectiveness *in vitro* and *in vivo*. In the first experiments, high titer verotoxin antibodies were generated in laying hens hyperimmunized with chemically detoxified and/or native verotoxins. These Laying hens were immunized with either recombinant *E. coli* O157:H7 VT1 or VT2 (rVT1 and rVT2) treated with glutaraldehyde and mixed with adjuvant.

Next, toxin-reactive polyclonal antibodies were isolated by bulk fractionation from egg yolks pooled from hyperimmunized hens. Large quantities of polyclonal antibodies ($\lg Y$) were harvested from resulting eggs using a two-step polyethylene glycol fractionation procedure.

Third, the immunoreactivity and yields of VT lgY were analyzed by analytical immunochemical methods (e.g., enzyme immunoassay (EIA) and Western blotting). EIA and Western blot analysis showed that the resulting egg preparations contained high titer IgY that reacted with both the immunizing and the heterologous toxins (i.e., rVT1 IgY reacted against both rVT1 and rVT2, and vice versa).

Fourth. VT neutralization potency was analyzed *in vitro* using a Vero cytotoxicity assay. Vero cytotoxicity of rVT1 and rVT2 could be completely inhibited by VT IgY. These antibodies also demonstrated substantial verotoxin cross-neutralization.

Fifth, the efficacy of passively administered avian verotoxin antibodies in preventing the lethal effects of verotoxin poisoning was assessed in a mouse disease model. Toxin neutralizing antibodies were administered by parenteral dosing regimens to assess the most

effective strategy for therapeutic intervention. Efficacy of verotoxin antibodies was demonstrated using multiple murine disease models. In these experiments, antibodies prevented both the morbidity and lethality of homologous and heterologous toxins using a toxin/antitoxin premix format: mice infected orally with a lethal dose of viable *E. coli* O157:H7 were protected from both morbidity and lethality when treated parenterally four hours post-infection with either rVT1 or rVT2 antibodies: and mice given a lethal dose of *E. coli* O91:H21 (a particularly virulent strain which only produces VT2c, a VT2 structural variant) and treated parenterally *up to 10 hours later* with rVT1 lgY administered parenterally were protected from both morbidity and lethality.

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EXAMPLE 1

TOXIN ANALYSIS AND IMMUNIZATION

Purified recombinant *E. coli* O157:H7 verotoxins, rVT1 and rVT2, were obtained from Denka Sieken Co., Ltd. (Tokyo, Japan). Toxin genes were isolated, inserted into expression plasmids, and expressed in *E. coli*. Recombinant proteins were then purified by ammonium sulfate precipitation, ion exchange chromatography on DEAE Sephacryl and hydroxyapatite, and gel filtration chromatography by the supplier. Upon receipt, toxins were analyzed to verify identity, purity and toxicity, as described below.

20 A. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Samples of each toxin (2 μg) were heat-denatured in a buffer containing SDS and β-mercaptoethanol followed by electrophoresis on 10–20% gradient gels (Bio-Rad. Richmond. CA). Resolved polypeptide bands were visualized using the silver stain procedure of C.R. Merril. *et al.*. "Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins." Science 211: 1437-1438 (1981).

VT1 and VT2 are each composed of subunit Λ and multiple copies of subunit B. Subunit A is often nicked into fragments A1 and A2 which are linked by a disulfide bridge. As shown in Figure 1, when separated by SDS-PAGE in the presence of β-mercaptoethanol. rVT1 resolved into 3 bands that corresponded to subunit A (~31 Kda), fragment A1 (~27 Kda) and a mixture of subunit B and fragment A2 (~4 Kda). Similarly, rVT2 resolved into subunit A (~33 Kda), fragment A1 (~27 Kda) and a mixture of subunit B and fragment A2 (~8 Kda) (Figure 1). In this Figure, rVT1 is in Lane 1, and rVT2 is in Lane 2; the positions of

molecular weight markers (Kda) are shown at the left. VT component polypeptides are identified at the right.

These results are consistent with previous reports of VT1 and VT2 purified from naturally occurring toxigenic strains (V. V. Padhye et al., "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From Escherichia coli O157:H7." Biochem. Biophys. Res. Commun., 139: 424-430 [1986]; and F. B. Kittel et al., "Characterization and inactivation of verotoxin 1 produced by Escherichia coli O157:H7." J. Agr. Food Chem., 39: 141-145 [1991]).

10 B. High Performance Liquid Chromatography (HPLC).

Chromatography was performed at room temperature (RT) under isocratic conditions using a Waters 510 HPLC pump. Eluted protein was measured using a Waters 490E programmable multi-wavelength detector (Millipore Corp., Milford, MA). The VT's were separated on an 8 x 300 mm (ID) Shodex KW803 column, using 10 mM sodium phosphate. 0.15 M NaCl. pH 7.4 (phosphate buffered saline [PBS]) as the mobile phase at a flow rate of 1 ml/min.

The purity of non-denatured rVT's was assessed by HPLC. As shown in the chromatographs in Figure 2, each toxin eluted at approximately 10 min, as a single absorbance peak at 280 nm. By integration of the area under each peak, the rVT's were shown to be >99% pure.

C. Vero Cell Cytotoxicity Assay.

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Cytotoxic activity of rVT1 and rVT2 was assessed using modified procedures of Padhye. et al. (V. V. Padhye et al., "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From Escherichia coli O157:H7." Biochem. Biophys. Res. Commun., 139: 424-430 [1986]), and McGee. et al., (Z. A. McGee. et al., "Local induction of tumor necrosis factor as molecular mechanism of mucosal damage by gonococci." Microbial Pathogenesis 12: 333-341 [1992]). Microtiter plates (96 well. Falcon. Microtest III) were inoculated with approximately 1 x 10⁴ Vero cells (ATCC. CCL81) per well (100 µl) and incubated overnight at 37°C in the presence of 5% CO₂ to form Vero cell monolayers. rVT1 and rVT2 solutions were serially diluted in Medium 199 supplemented with 5% fetal bovine serum (Life Technologies. Grand Island, NY), added to each well of the microtiter plates and incubated at 37°C for 18–24 hrs. Adherent (viable) cells were stained with 0.2% crystal

violet (Mallinckrodt) in 2% ethanol. Excess stain was rinsed away and the stained cells were solubilized by adding 100 µl of 1% SDS to each well. Absorbance of each well was measured at 570 nm, and the percent cytotoxicity of each test sample was calculated using the following formula:

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% Vero Cytotoxicity = [1 - (Absorbance Sample/Absorbance Control)] x 100

To determine whether the rVT's possessed potency equivalent to published cytotoxicity values, a Vero cell cytotoxicity assay was performed (Figure 3). Between 0.01-10.000 pg of either rVT1 or rVT2 was added to Vero cells. The amounts of rVT causing 50% cell death (CD₅₀), as calculated by second degree polynomial curve fitting were 0.97 pg and 1.5 pg, for rVT1 and rVT2, respectively. These results are consistent with CD50 values reported previously for naturally occurring VT1 and VT2 in the range 1-35 pg and 1-25 pg. respectively (M. Petric et al., Purification and biological properties of Escherichia coli verocytotoxin." FEMS Microbiol. Lett., 41: 63-68 [1987]: V. L. Tesh, et al., "Comparison of relative toxicities of Shiga-Like toxins Type I and Type II for mice." Infect. Immun., 61: 3392-3402 [1993]: N. Dickie et al.. "Purification of an Escherichia coli Serogroup O157:H7 verotoxin and its detection in North American hemorrhagic colitis isolates." J. Clin. Microbiol., 27: 1973-1978 [1989]; and U. Kongmuang. et al.. "A simple method for purification of Shiga or Shiga-Like toxin from Shigella dysemeriae and Escherichia coli O157:H7 by immunoaffinity chromatography," FEMS Microbiol. Lett., 48: 379-383 [1987]). It has been observed that toxicity is lost with storage, explaining why higher amounts of toxin were used in the neutralization assays described below.

D. Mouse Lethal Dose Determination.

To verify rVT1 and rVT2 toxicity, male (20–22 g) CD-1 mice were injected intraperitoneally with varying amounts of rVT1 or rVT2 in 200 μ L phosphate buffer. Doses were selected based on published LD₅₀ values for VT1 and VT2 in CD-1 mice. To minimize the sacrifice of live animals, a full statistical toxin LD₅₀ was not determined. Mice were observed for morbidity and mortality over 7-day period.

Further confirmation of rVT toxicity was obtained from mouse lethality experiments (Table 2). Mice were injected intraperitoneally with varying amounts of either rVT1 or rVT2 and observed 7 days for mortality. Within 72–120 hrs. post-injection, all of the mice died

from 100 ng of rVT1 or 10 ng of rVT2, respectively. This lethality study served as a verification of expected toxicity but not as a statistical determination of LD₅₀. Nonetheless, these results were consistent with toxicity studies which reported LD₅₀ values in CD-1 mice of 0.4–2.0 µg for purified VT1 and 0.001–1.0 µg for purified VT2 (V. L. Tesh, *et al.*, "Comparison of relative toxicities of Shiga-Like toxins Type I and Type II for mice," Infect. Immun., 61: 3392-3402 [1993]; and A. D. O'Brien, and G. D. LaVeck, "Purification and characterization of *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*," Infect. Immun., 40: 675-683 [1983]).

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Table 2. Lethality of rVT1 in CD-1 Mice

ng VT1 Injected	Survivors/Total	Hours Post-Injection
	7 /7	24 ± 2
100	5/7	48 ± 2
	0/7	72 ± 2
10	7/7	24 ± 2
	7/7	48 ± 2
	7/7	72 ± 2
1.0	6/6	24 ± 2
	6/6	48 ± 2
	6/6	72 ± 2

Table 3. Lethality of rVT2 in CD-1 Mice

ng VT2 Injected	Survivors/Total	Hours Post-Injection
. 10	3/6	48 ± 2
	2/6	72 ± 2
	0/6	120 ± 2
1.0	5/6	48 ± 2
	4/6	72 ± 2
	0/6	120 ± 2
0.1	6/6	48 ± 2
	6/6	72 ± 2
	6/6	120 ± 2

The recombinant toxins used in these studies thus appeared to contain protein components and toxicities consistent with literature reports for native toxins. Based on these structural and functional analyses, the rVT's were considered suitable as antigens to generate specific avian antibodies.

20 E. Antigen Preparation.

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Lyophilized samples, rVT1 and rVT2 were received and each was reconstituted with 2.5 mL of deionized water to a final concentration of 100 µg/ml in phosphate buffer. To form a toxoid, the solutions were then treated with 0.4% glutaraldehyde (Mallinckrodt) at 4°C overnight and stored at -20°C thereafter. When needed, toxoid was thawed and mixed 5:1 (volume:volume) with GERBU adjuvant (C. C. Biotech Corporation, Poway, CA). White Leghorn laying hens were injected subcutaneously with 25 µg of either rVT1 or rVT2 toxoid in adjuvant at 2–3 week intervals.

EXAMPLE 2 PEG EXTRACTION OF EGG YOLK ANTIBODY

Hyperimmune eggs were collected after 3 immunizations with toxoid. Egg yolks were separated from whites, pooled according to their immunogen group and blended with 4 volumes of 10 mM sodium phosphate. 150 mM NaCl. pH 7.4 (PBS). Polyethylene glycol

8000 (PEG) (Amresco, Solon, OH) was then added to a final concentration of 3.5% and the mixture centrifuged at 10,000 x g for 10 min, to remove the precipitated lipid fraction. IgY-rich supernatant was filtered through cheesecloth and PEG was again added to a final concentration of 12%. The solution was centrifuged as above and the resulting supernatant discarded. The IgY pellet was then dissolved in PBS to either the original (1X PEG IgY) or 1/4 of the original (4X PEG IgY) yolk volume, filtered through a 0.45 µ membrane and stored at 4°C.

EXAMPLE 3 ANTITOXIN IMMUNOASSAYS

A. Enzyme Immunoassay (EIA).

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EIA was used to monitor antibody responses during the immunization course. Wells of 96-well Pro-Bind microtiter plates (Falcon, through Scientific Products, McGaw Park, IL) were each coated with 1 μg of rVT's (not toxoid) in PBS overnight at 2–8°C. Wells were washed 3 times with PBS containing 0.05% Tween-20 (PBS-T) to remove unbound antigen, and the remaining protein binding sites were blocked with PBS containing 1 mg/ml BSA for 60 min, at room temperature (RT), IgY, diluted in PBS, was then added to the wells and incubated for 1 hr. at 37°C. Wells were washed as before to remove unbound primary antibody and incubated for 1 hr. at 37°C with alkaline phosphatase-conjugated rabbit-antichicken IgG (Sigma Chemical Company, St. Louis, MO) diluted 1:1000 in PBS-T. Wells were again washed and 1 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Company, St. Louis, MO) in 50 mM Na₂CO₃, 10 mM MgCl₂, pH 9.5 was added and allowed to incubate at RT. Phosphatase activity was detected by absorbance at 410 nm using a Dynatech MR700 microtiter plate reader.

Laying Leghorn hens were immunized as described above (Example 1, part E), using glutaraldehyde-treated rVT's. Following several immunizations, eggs were collected and IgY harvested by PEG fractionation. Figures 4 and 5 show rVT1 or rVT2 specific antibody responses detected using ElA at dilutions of the original yolk IgY concentration of 1:30,000 and 1:6,000, respectively. IgY fractionated similarly from unimmunized hens (i.e., preimmune antibody) did not react with either antigen at test dilutions above 1:50. Although these ElA results indicate significant antibody responses, prior experience with other toxin antigens has shown that optimization of immunization regimens, including increasing the amount of

antigen, can yield titers in excess of 1:100.000 (B. S. Thalley, et al., "Development of an Avian Antitoxin to Type A Botulinum Neurotoxin," in Botulinum and Tetanus Neurotoxins:

Neurotransmission and Biomedical Aspects. B. R. DasGupta, (ed.) [Plenum Press, New York, 1993] pp. 467-472). As may be expected due to their structural homology and consistent with previous reports (e.g., V. V. Padhye et al., "Production and characterization of monoclonal antibodies to verotoxins 1 and 2 from Escherichia coli O157:H7." J. Agr. Food Chem., 39: 141-145 [1989]; S. C. Head et al., "Purification and characterization of verocytotoxin 2."

FEMS Microbiol, Lett., 51: 211-216 [1988]; and N. C. Strockbine et al., "Characterization of Monoclonal Antibodies against Shiga-Like Toxin from Escherichia coli," Infect. Immun., 50: 695-700 [1985]). Figures 4 and 5 also demonstrate that antibodies generated against one toxin cross-reacted in vitro with the other toxin.

B. Western Blot Analysis.

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Western blots (Figure 6) performed to determine the reactivity of rVT antibodies against constituent VT polypeptides showed that rVT1 and rVT2 antibodies reacted with subunit A and fragment A1 of either toxin, and with subunit B and fragment A2 of rVT1 only. In this Figure, Panel A contains preimmune lgY, Panel B contains rVT1 lgY, and Panel C contains rVT2 lgY. Lane 1 in each panel contains rVT1 (2µg) and Lane 2 contains rVT2 (2 µg). Preimmune lgY was largely nonreactive to either rVT. Both rVT lgY preparations, however, failed to react with subunit B and fragment A2 of rVT2. Some explanations for this lack of measurable reactivity might include poor immunogenicity, denaturation of the immunogen during glutaraldehyde treatment, loss of conformational epitopes due to detergent or reducing agent, or poor transfer to nitrocellulose.

To resolve the high and low molecular weight components. 2 µg each of rVT1 and rVT2 were separated by SDS-PAGE (described above) and then transferred to nitrocellulose paper using the Milliblot-SDE system (Millipore, Medford, MA) according to the manufacturer's instructions. Paper strips were stained temporarily with Ponceau S (Sigma Chemical Company, St. Louis, MO) to visualize the polypeptides and then blocked overnight in PBS containing 5% dry milk. Each strip was agitated gently in IgY diluted in PBS-T for 2 hrs. at RT. Strips were each washed with three changes of PBS-T to remove unbound primary antibody and incubated for 2 hrs. at RT with goat anti-chicken alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, MD) diluted 1:500 in PBS-T containing 1 mg/ml BSA. The blots were washed as before and rinsed in 50 mM Na₂CO₃, pH 9.5. Strips were

submerged in alkaline-phosphatase substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (Kirkegaard and Perry) until sufficient signal was observed. Color development was stopped by flooding the blots with water.

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EXAMPLE 4

IN VITRO TOXIN NEUTRALIZATION: VERO CELL ASSAY

IgY neutralization of rVT1 and rVT2 was assessed using the modified Vero cytotoxicity assay described above (Example 1, part C). Various concentrations of IgY, diluted in Medium 199 supplemented with 5% fetal bovine serum (GIBCO), were mixed with sufficient toxin to cause 50% cell death and allowed to incubate at 37°C for 60 minutes. These toxin/antibody mixtures were then added to Vero cell-coated microtiter plate wells according to the procedure described above (Example 1, part C).

The toxin neutralization capacity of the rVT antibodies was analyzed first using a Vero cell toxicity assay. The results in Figure 7 show that rVT1 IgY neutralized completely the cytotoxic activity of rVT1 at an endpoint dilution of 1/320. Furthermore, rVT2 IgY neutralized the heterologous rVT1 toxin, but at a higher endpoint concentration.

In a similar experiment (see Figure 8), rVT1 and rVT2 antibodies were each able to neutralize rVT2 at equivalent endpoint dilutions. This strong cross-neutralization correlates with the observed strong cross-reactivity of VT1 IgY with VT2 A seen on Western blots (Figure 6). These results show that IgY antibodies are able to neutralize effectively VT cytotoxicity and that the antibodies can cross-neutralize structurally-related heterologous toxins.

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EXAMPLE 5

TOXIN NEUTRALIZATION: MOUSE ASSAYS

A. Toxin Challenge Model.

IgY in PBS was premixed with a lethal dose of toxin (as determined above) and injected intraperitoneally into male CD-1 (20–22 gm) mice. Mice were observed for a 7-day period for signs of intoxication such as ruffled fur. huddling and disinclination to move. followed by hind leg paralysis, rapid breathing and death. Untreated, infected mice usually died within 12 hrs. after signs of severe illness (i.e., within 48–72 hrs. post-injection).

Once it was demonstrated that rVT antibodies were able to neutralize rVT cytotoxicity in vitro, protection experiments were next performed in mice. First, animals were challenged with rVT premixed with rVT IgY to determine whether toxin lethality could be neutralized under conditions optimal for antigen/antibody reaction. Tables 4 and 5 show that antibodies premixed with the homologous toxin (e.g., rVT1 with rVT1 lgY) prevented lethality of rVT. Preimmune lgY was unable to neutralize either toxin in these studies.

Table 4
Neutralization of rVT1 Using rVT lgY

100 ng rVT2 Premixed*	Survivors/Total	n
Preimmune Antibody	0/12	P
rVT1 Antibody	12/12	< 0.001
rVT2 Antibody	12/12	< 0.001

*Toxin was pre-mixed with IgY and incubated for 1 hour at room temperature prior to administration.

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Table 5

Neutralization of rVT2 Using rVT IgY

10 ng rVT1 Premixed*	Survivors/Total	n
Preimmune Antibody	0/12	1
rVT1 Antibody	12/12	< 0.001
rVT2 Antibody	12/12	< 0.001

25 *Toxin was pre-mixed with IgY and incubated for 1 hour at room temperature prior to administration.

Antibodies premixed with the heterologous toxin (e.g., rVT2 with rVT1 IgY) also prevented lethality in vivo. These data are in contrast to previous observations where rabbit polyclonal antibodies generated against either toxin were cross-reactive with the heterologous toxin by EIA and Western blot, but were unable to neutralize the heterologous toxin in either Vero cell cytotoxicity and mouse lethality assays (S. C. Head, et al., "Serological differences between verocytotoxin 2 and Shiga-like toxin II," Lancet ii: 751 [1988]; S. C. Head et al., "Purification and characterization of verocytotoxin 2," FEMS Microbiol, Lett., 51: 211-216

[1988]; N. C. Strockbine et al., "Characterization of Monoclonal Antibodies against Shiga-Like Toxin from Escherichia coli," Infect. Immun., 50: 695-700 [1985]; and V. V. Padhye et al., "Purification and Physicochemical Properties of a Unique Vero Cell Cytotoxin From Escherichia coli O157:H7." Biochem. Biophys. Res. Commun., 139: 424-430 [1986]).

However, Head *et al.*, showed that VT2 B-subunit specific monoclonal antibodies neutralized VT1 weakly in a Vero cytotoxicity assay (S. C. Head. *et al.*, "Serological differences between verocytotoxin 2 and Shiga-like toxin II," Lancet ii: 751 [1988]). In a report by Donohue-Rolfe, *et al.*, a VT2 B subunit-specific monoclonal antibody neutralized both VT1 and VT2 completely in a Hela cytotoxicity assay (A. Donohue-Rolfe *et al.*, "Purification of Shiga toxin and Shiga-like toxins I and II by receptor analog affinity chromatography with immobilized P1 glycoprotein and production of cross reactive monoclonal antibodies." Infect. Immun., 57: 3888-3893 [1989]).

These results showed for the first time complete cross-neutralization in Vero cell cytotoxicity and mouse lethality assays, revealing that VT1 and VT2 do indeed share common neutralizing epitopes. These results may indicate that hens generate different antibody specificities as compared to mammals, and/or that differences in immunization methods might have maintained the immunogenicity of conformational epitopes necessary for cross-neutralization. Nonetheless, this cross-neutralization suggests that IgY antibodies may contain the range of reactivities essential for an effective antitoxin.

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B. Viable organism infection model.

Streptomycin-resistant *E. coli* O157:H7 (strain 933 cu-rev) or *E. coli* O91:H21 (strain B2I1) (both kindly provided by Dr. Alison O'Brien, Dept. of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD) were used in a murine infection model described by Wadolkowski, *et al.* (E. A. Wadolkowski *et al.*, "Mouse model for colonization and disease caused by enterohemorrhagic *Escherichia coli* O157:H7." Infect. Immun., 58: 2438-2445 [1990]). Organisms were grown in Luria broth and incubated overnight at 37°C in an Environ Shaker (Lab Line, Melrose Park, IL) (T. Maniatis *et al.*, Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., [1982]). Bacterial suspensions were centrifuged at 6700 x g for 5 minutes. The resulting pellet was then washed twice with sterile PBS and resuspended in sterile 20% (w/v) sucrose. Five to 8 week-old male CD-1 mice were provided drinking water containing 5 mg/ml streptomycin sulfate *ad libitum* for 24 hrs. Food and water were then withheld for

another 16–18 hrs. after which mice were challenged orally with 10¹⁰ streptomycin-resistant *E. coli* O157:H7 or O91:H21. Mice were housed individually and permitted food and water containing 5 mg/ml streptomycin sulfate. IgY was injected intraperitoneally at varying times post-infection and animals observed for both morbidity and mortality for 10 days.

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To monitor bacterial colonization in animals, I gram of feces was collected. homogenized, and plated onto MacConkey agar medium (Difco Laboratories, Detroit, MI) containing 100 µg/ml streptomycin and incubated at 37°C as described by Wadolkowski, et al. (E. A. Wadolkowski et al., "Mouse model for colonization and disease caused by enterohemorrhagic Escherichia coli O157:H7." Infect, Immun., 58: 2438-2445 [1990]). The serotype of E. coli O157:H7, 933 cu-rev excreted in feces was confirmed by slide agglutination with O- and H-specific antisera (Difco Laboratories, Detroit, MI).

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Kidneys were removed from experimental animals and fixed in 10% buffered neutral formalin. Sections of parafilm-embedded tissue were stained with hematoxylin and eosin (General Medical Laboratories, Madison. WI) and examined by light microscopy. All tissue sections were coded to avoid bias before microscopic examination to determine renal pathology.

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The toxin neutralization ability of rVT IgY was further studied using a streptomycintreated CD-1 mouse infection model. This model was chosen because it produces definitive systemic pathology and reproducible mortality.

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In contrast to previous studies by Wadolkowski. et al. (E. A. Wadolkowski et al.. "Acute renal tubular necrosis and death of mice orally infected with Escherichia coli strains that produce Shiga-like toxin Type II." Infect. Immun. 58: 3959-3965 [1990]). where mice were given subunit-specific monoclonal antibodies prior to infection, the mice in this study were inoculated orally with 2 x 10¹⁰ viable E. coli O157:H7 (strain 933 cu-rev) and treated with rVT IgY 4 hrs. following inoculation. Fecal cultures showed that 10⁷-10⁸ challenge organisms per gram of feces were shed throughout the course of the experiment, thus confirming that infection was established. Tables 6 and 7 show that animals treated with either rVT1 or rVT2 IgY were protected from lethality caused by infection (p<0.01 and p<0.001, respectively) and that preimmune IgY failed to provide protection to the mice.

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Table 6
Protection of Mice From E. coli O157:H7
With rVT1 IgY

IgY Treatment	Survivors/Total	p	Morbidity/Total
Preimmune Antibody	0/5		5/5
rVT1 Antibody	9/10	< 0.01	1/10

^{*}IgY was administered intraperitoneally 4 hours following infection, and once daily for 10 days thereafter.

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Table 7
Protection of Mice From E. coli O157:H7
With rVT2 lgY

IgY Treatment	Survivors/Total	p	Morbidity/Total
Preimmune Antibody	0/6		6/6
rVT2 Antibody	10/10	< 0.005	0/10

*lgY was administered intraperitoneally 4 hours following infection, and once daily for 10 days thereafter.

Renal histopathology (see Figure 9) of the control (preimmune IgY) animals showed dilation, degeneration and renal tubular necrosis with no glomerular damage. This is consistent with previous reports showing that renal tubular involvement occurs predominantly in this streptomycin-treated mouse infectivity model (E. A. Wadolkowski et al., "Acute renal tubular necrosis and death of mice orally infected with Escherichia coli strains that produce Shiga-like toxin Type II." Infect. Immun., 58: 3959-3965 [1990]). Importantly, none of the survivors exhibited similar signs of morbidity though treated with IgY 4 hrs. after infection (see Figure 9).

Furthermore, avian antibodies generated against rVT1 were able to prevent both mortality and morbidity in a mouse model where VT2 alone is implicated in the pathogenesis and lethality of *E. coli* O157:H7 strain 933 cu-rev (E. A. Wadolkowski *et al.*, "Acute renal tubular necrosis and death of mice orally infected with *Escherichia coli* strains that produce Shiga-like toxin Type II." Infect. Immun., 58: 3959-3965 [1990]).

To assess the broader utility of the IgY verotoxin antibodies in treating VTEC-associated disease, the mouse infectivity study was performed using a more virulent VTEC serotype known to produce VT2c—a structural variant of VT2—but not VT1 (S. W. Lindgren

et al., "Virulence of enterohemorrhagic Escherichia coli O91:H21 clinical isolates in an orally infected mouse model." Infect. Immun., 61: 3832-3842 [1993]).

Mice were inoculated orally with 5 x 10^9 E. coli O91:H21 (strain B2F1) and treated subsequently with IgY. Notably, the heterologous rVT1 IgY protected strongly against the lethal effects of the VT2c structural variant, even when administered as long as 10 hrs. following infection (Table 8). Ten hours was the longest treatment window tested in this study. Only 1 of the 8 animals treated with rVT1 IgY died (p <0.02), and those that survived showed no overt signs of renal histopathology (i.e., acute bilateral tubular necrosis). It can thus be concluded that rVT1 IgY completely neutralized toxicity of VT2c, indicating its potential as a therapeutic for at least one other pathogenic VTEC.

Table 8
Protection of Mice From E. coli O91:H21
With rVT1 IgY

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IgY Treatment	Survivors/Total	р	Morbidity/Total
Preimmune Antibody	0/7		7/7
rVT1 Antibody	7/8	< 0.02	1/8

*IgY was administered intraperitoneally 10 hours following infection, and once daily for 8 days thereafter.

These Examples highlight several important findings supporting the feasibility of using verotoxin antitoxin. First, polyclonal IgY generated against either VT1 or VT2 from *E. coli* O157:H7, cross-reacted with and fully cross-neutralized the toxicity of the non-immunizing toxin both *in vitro* and *in vivo*. Second, recombinant toxins fully neutralized the toxicity of naturally-occurring toxins produced by *E. coli* O157:H7 during the course of infection. Third, antibodies generated against rVT1 from *E. coli* O157:H7 could prevent morbidity and mortality in mice infected orally with lethal doses of *E. coli* O91:H21, a particularly virulent strain which only produces VT2c, suggesting their utility in preventing systemic sequelae. Because VT1 is identical to Shiga-toxin (A. D. O'Brien *et al.*, "Shiga and Shiga-like toxins. Microbial Rev., 51: 206-220 [1987]), VT antibodies may also be useful in preventing complications stemming from *Shigella dysenteriae* infection. Finally, animals treated with VT

IgY were protected against both death and kidney damage when treated as long as 10 hrs. after infection, supporting the hypothesis that a window for antitoxin intervention exists.

These studies strongly support the use of parenterally-administered, toxin-specific IgY as a antitoxin to prevent life-threatening complications associated with *E. coli* O157:H7 and other VTEC infections. It is contemplated that this approach would be most useful in preventing HUS and other complications when administered after the onset of bloody diarrhea and before the presentation of systemic disease.

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The VT IgY developed in these studies were shown to react with and neutralize both recombinant and naturally-occurring VT. The antibody titers as measured by EIA are indicative of reasonable antibody production in the hen, however much higher production levels can be obtained with larger immunizing doses.

The results from these Examples clearly demonstrate the feasibility and provide the experimental basis for development of an avian antidote for E. coli O157:H7 verotoxins suitable for use in humans. In contrast to previous reports showing that rabbit polyclonal VT1 and VT2 antibodies cross-reacted, but did not cross-neutralize the heterologous toxin in Vero cytotoxicity or in mouse lethality studies (e.g., V. V. Padhye et al., "Production and characterization of monoclonal antibodies to verotoxins 1 and 2 from Escherichia coli O157:H7," J. Agr. Food Chem., 39: 141-145 [1989]; S. C. Head et al., "Purification and characterization of verocytotoxin 2." FEMS Microbiol. Lett., 51: 211-216 [1988]; and N. C. Strockbine et al., "Characterization of monoclonal antibodies against Shiga-like toxin from Escherichia coli," Infect. Immun., 50: 695-700 [1985]), these data provide the first demonstration of cross-neutralization in vivo. Antibodies against one toxin neutralized completely the heterologous toxin in both Vero cytotoxicity and mouse lethality assays. Both rV71 and rVT2 antibodies also prevented morbidity (as assessed by renal histopathology) and mortality in mice infected with lethal doses of E. coli O157:H7 - the etiologic agent in 90% of the documented cases of hemolytic uremic syndrome (HUS) in the U.S. (P. M. Griffin and R. V. Tauxe. "The epidemiology of infections caused by Escherichia coli O157:H7, other enterohemorrhagic E. coli, and the associated hemolytic uremic syndrome," Epidemiol, Rev., 13: 60 [1990]). With at least two other VTEC serotypes known to cause HUS, the finding that rVT1 antibodies neutralized a VT2 variant produced by E. coli O91:H21 suggests that avian polyclonal antibodics may provide an effective antidote against other verotoxinproducing E. coli. These data also show for the first time, that antibodies may be administered after infection and still protect against morbidity and mortality.

EXAMPLE 6

EXPRESSION OF TOXIN GENES

The previous Examples clearly showed that avian polyclonal antibodies to recombinant toxins protected animals infected with verotoxigenic *E. coli*. This Example includes expression of toxin genes (A and B subunits alone and together as whole toxins) in suitable prokaryotic expression systems to achieve high levels of VT antigen production.

The sequence of the toxin gene has been determined (see e.g., M.P. Jackson et al., "Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from Escherichia coli 933." 44:109 [1987]). The coding regions of the A and B subunits of VT-1 are listed in SEQ ID NOS:1 and 3. respectively. The corresponding amino acid sequence of the A and B subunits of the VT-1 toxin are listed in SEQ ID NOS:2 and 4. respectively. The coding regions of the A and B subunits of VT-2 are listed in SEQ ID NOS:5 and 7. respectively. The corresponding amino acid sequence of the A and B subunits of the VT-2 toxin are listed in SEQ ID NOS:6 and 8. respectively. In addition, SEQ ID NOS:9 and 10 list the sequences which direct the expression of a poly-cistronic RNA capable of directing the translation of both the A and B subunits from the VT-1 and VT-2 genes, respectively.

In choosing a strategy for recombinant VT antigen production, there are three primary technical factors to consider. First, the appropriate VT antigen components representing the spectrum of toxin epitopes encountered in nature must be utilized. Second, the protein antigens must be expressed at sufficient levels and purity to enable immunization and large-scale antibody purification. Third, the neutralizing epitopes must be preserved in the immunogen and immunoabsorbant. Approaches that offer the greatest promise for high level expression of periplasmically localized, native, affinity-tagged proteins were developed. Figure 10 shows the fusion constructs of VT components and affinity tags.

A. Expression of affinity-tagged C-terminal constructs.

The VT1 and VT2 A and B subunits (SEQ ID NOS:1, 3, 5 and 7) are cloned into the pET-23b vector (Novagen). This vector is designed to allow expression of native proteins containing C-terminal poly-His tags. The vector utilizes a strong T7 polymerase promoter to drive high level expression of target proteins. The methionine initiation codon is engineered to contain a unique *Ndel* restriction enzyme site (CATATG). The VT1 and VT2 genes are engineered to convert the signal sequence methionine codon into a *Ndel* site utilizing PCR

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mutagenesis. PCR primers were designed which contain the sequence GCCAT fused to the first 20–24 bases of the genes (starting at the ATG start codon of the signal tag; SEQ ID NOS:12-19, see Table below). Upon PCR amplification, the 5° start codon of each gene is converted to an NdeI site, compatible with the pET-23 vector-encoded NdeI site, allowing cloning of the amplified genes into the vector without the addition of vector-encoded amino acids.

Primers containing the C-terminal 7 codons of each gene (21 bases) fused to the sequence CTCGAGCC were synthesized, in order to add a C-terminal poly-His tag to each gene. The underlined bases are an XhoI site, that is compatible with the XhoI site of the pET-23 vector. These primers precisely delete the native stop codons, and when cloned into the pET-23 vector, add a C-terminal extension of "LeuGluHisHisHisHisHisHisHis" (SEQ ID NO: 11). The following table lists the primer pairs are utilized to create PCR fragments containing the A and B subunits derived from VT-1 and VT-2 toxin genes suitable for insertion into the pET-23b vector.

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Table 9
Primers

Toxin Gene and Subunit	N-terminal Primer	C-terminal Primer
VT-1 Subunit A	SEQ ID NO:12	SEQ ID NO:13
VT-1 Subunit B	SEQ ID NO:14	SEQ ID NO:15
VT-2 Subunit A	SEQ ID NO:16	SEQ ID NO:17
VT-2 Subunit B	SEQ ID NO:18	SEQ ID NO:19
VT-1 Subunits A and B	SEQ ID NO:12	SEQ ID NO:15
VT-2 Subunits A and B	SEQ ID NO:16	SEQ ID NO:19

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Thus, utilizing PCR amplification with the above modified N- and C-terminal primers, the A and B subunits of VT1 and VT2 are expressed as proteins containing an 8 amino acid C-terminal extension bearing an poly-histidine affinity tag. The amino acid sequence of the histidine-tagged VT-1 A subunit produced by expression from the pET-23b vector is listed in SEQ ID NO:21 (the associated DNA sequence is listed in SEQ ID NO:20): the amino acid sequence of the histidine-tagged VT-1 B subunit is listed in SEQ ID NO:23 (the associated

DNA sequence is listed in SEQ ID NO:22); the amino acid sequence of the histidine-tagged VT-2 A subunit is listed in SEQ ID NO:25 (the associated DNA sequence is listed in SEQ ID NO:24); the amino acid sequence of the histidine-tagged VT-2 B subunit is listed in SEQ ID NO:27 (the associated DNA sequence is listed in SEQ ID NO:26).

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Both subunits may be expressed from a single expression constructs by utilizing SEQ ID NOS:12 and 15 to prime synthesis of the VT-1 toxin gene and SEQ ID NOS:16 and 19 to prime synthesis of the VT-2 toxin gene. The resulting PCR products are cleaved with *NdeI* and *XhoI*, as described for the cloning of the subunit genes into the pET-23b vector. Expression of the A and B subunits from such an expression vector, results in the expression of a native A subunit and a his-tagged B subunit. As the A and B subunits assemble into a complex, the presence of the his-tag on only the B subunit is sufficient to allow purification of the holotoxin on metal chelate columns as described below.

The proofreading *Pfii* polymerase (Stratagene) is utilized for PCR amplification to reduce the error rate during amplification. Genomic DNA from an *E. coli* O157:H7 strain is utilized as template DNA. Following the PCR, the amplification products are digested with *Ndel* and *Xhol* and cloned into the pCR-Script SK cloning vehicle (Stratagene) to permit DNA sequence analysis of the amplified products. The DNA sequence analysis is performed to ensure that no base changes are introduced during amplification. Once the desired clones are identified by DNA sequencing, the inserts are then excised utilizing *Ndel* and *Xhol*, and cloned into a similarly cut pET-23b vector to create the expression constructs. According to the published sequences, neither the VT1 nor VT2 genes contain either of these restriction sites.

The poly-His-tagged proteins produced by expression of the VT-1 and VT-2 gene sequences in the pET-23b constructs are then purified by IMAC. This method uses metal-chelate affinity chromatography to purify native or denatured proteins which have histidine tails (see e.g., K. J. Petty, "Metal-Chelate Affinity Chromatography." in Current Protocols in Molecular Biology, Supplement 24, Unit 10.11B [1993]).

B. Expression of Toxin Containing N-terminal Affinity Tags

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Two expression systems, pMal-p2 and pFLAG-1 are utilized to attach an N-terminal affinity tag to the A subunits from the VT-1 and VT-2 toxins.

MBP-tagged constructs. To construct A chains containing the maltose binding protein (MBP) at the N-terminus of the A subunit, PCR amplified gene products are cloned into the

pMal-p2 vector (New England Biolabs) as C-terminal fusions to a periplasmically-secreted version of the MBP. The MBP selectively binds to amylose resins and serves as an affinity tag on the MBP/A subunit fusion protein. The pMal-p2 vector contains an engineered factor Xa cleavage site, which permits the removal of the affinity tag (i.e., MBP) from the fusion protein after purification.

The MBP/A subunit fusions are generated as follows. The VT1 and VT2 A subunits are PCR-amplified utilizing the following DNA primers. SEQ ID NOS:28-31: SEQ ID NOS:28 and 29 comprise the 5' and 3' primers, respectively, for the amplification of the VT1 A subunit; SEQ ID NOS:30 and 31 comprise the 5' and 3' primers, respectively, for the amplification of the VT2 A subunit. In both cases, the 5' or N-terminal primer contains the sequence CGGAATTC fused to the first codon of the mature polypeptide (rather than the start of the signal peptide, since the MBP signal peptide is utilized). These 5' primers contain an engineered *EcoRI* site that is not contained internally in either gene, that is compatible with the *EcoRI* site of the pMal-p2 vector. The 3' or C-terminal primers incorporate an *XhoI* site as described above for the generation of the His-tagged toxins, but in this case, the 3' primer is designed to include the natural termination codon of the A subunits.

The genes are amplified. cloned into pCR-Script SK, and sequenced as described above. The inserts are then excised with *Eco*RI and *Xho*I, and cloned into *Eco*RI/SalI-cleaved pMal-p2 vector (SalI and XhoI sites are compatible). This construct allows expression and secretion of the VT1 and VT2 A subunit genes as C-terminal fusions with MBP. The amino acid sequence of the MBP/VT-1A fusion protein is listed in SEQ ID NO:33 (the associated DNA sequence is listed in SEQ ID NO:32). The amino acid sequence of the MBP/VT-2A fusion protein is listed in SEQ ID NO:35 (the associated DNA sequence is listed in SEQ ID NO:35).

The resulting fusion proteins are then affinity purified on an amylose column and the bound fusion protein is eluted under mild conditions by competition with maltose. The MBP N-terminal-tagged A subunits are cleaved with factor Xa and the MBP is removed by chromatography on an amylose column. The resulting A subunits which contain a 4 amino acid N-terminal extension are then used as immunogens.

Flag tag constructs. In an alternative embodiment, the VT1 and VT2 A subunit genes are engineered to contain the "flag tag" through the use of the pFLAG-1 vector system. The flag tag is located between the *OmpA* secretion signal sequence and the authentic N-

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terminus of the target protein in the pFlag-1 vector. To construct N-terminal flag-tagged A chains, the *EcoRI/XhoI* A subunit PCR fragments (generated as described above for the MBP fusion proteins) are cloned into identically cleaved pFlag-1 vector (Eastman-Kodak), to produce an expression construct utilizing the *OmpA* signal peptide for secretion of A subunit fusion proteins containing the flag peptide at the N-terminus. After secretion, the periplasmic protein contains the N-terminal 8 amino acid flag tag, followed by 4 vector-encoded amino acids fused to the recombinant A subunit. The amino acid sequence of the flag tag/VT-1 A subunit fusion protein is listed in SEQ ID NO:37 (the associated DNA sequence is listed in SEQ ID NO:36). The amino acid sequence of the flag tag/VT-2 A subunit fusion protein is listed in SEQ ID NO:39 (the associated DNA sequence is listed in SEQ ID NO:38).

The flag tag fusion proteins are then purified by immunoaffinity chromatography utilizing a calcium-dependent monoclonal antibody (Antiflag M1: Eastman-Kodak). Mild elution of purified protein is achieved by chelating the calcium in the column buffer with ethylenediamine tetraacetic acid (EDTA).

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C. Evaluation of fusion construct expression.

The fusion constructs described above are expressed in *E. coli* strain BL21, or T7 polymerase-containing derivatives [e.g., BL21(DE3), BL21(DE3) pLysS, BL21(DE3)pLysE] (Novagen) for pET plasmids, and periplasmically-secreted recombinant protein purified by affinity chromatography. Recombinant proteins are analyzed for correct conformation by testing the following parameters:

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a) It is believed that the B subunit must associate into pentamers to be conformationally correct. This is assessed by reducing and native SDS-PAGE analyses of native and chemically-cross-linked proteins and sizing HPLC;

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b) It is believed that a properly folded A subunit is expected to retain its native enzymatic activity. This is tested by its capacity to inhibit protein synthesis in an *in vitro* toxicity assay;

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c) It is believed that *in vitro* toxicity of assembled recombinant holotoxin is compared to commercially available holotoxins to determine whether recombinant A and B subunits can assemble into functional holotoxin. The

purified N-terminal-tagged A subunits (after cleavage and purification from MBP or untreated flag-tagged proteins) are combined *in vitro* with the corresponding B chains, and their toxicity evaluated utilizing a quantitative microtiter cytotoxicity assay, such as that described by M.K. Gentry and M. Dalrymple, "Quantitative Microtiter Cytotoxicity Assay for *Shigella* Toxin." J. Clin. Microbiol., 12:361-366 (1980).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: OPHIDIAN PHARMACEUTICALS, INC.
- (ii) TITLE OF INVENTION: TREATMENT FOR VEROTOXIN-PRODUCING E.
- (iii) NUMBER OF SEQUENCES: 39
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: MEDLEN & CARROLL
 - (B) STREET: 220 MONTGOMERY STREET, SUITE 2200
 - (C) CITY: SAN FRANCISCO
 - (D) STATE: CALIFORNIA
 - (E) COUNTRY: UNITED STATES OF AMERICA
 - (F) ZIP: 94104
- (v) COMPUTER READABLE FORM:

 - (A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 (B) FILING DATE:

 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: CARROLL, PETER G.
 - (B) REGISTRATION NUMBER: 32,837
 - (C) REFERENCE/DOCKET NUMBER: OPHD-02171
 - (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (415) 705-8410
 - (B) TELEFAX: (415) 397-8338
- (2) INFORMATION FOR SEQ ID NO:1:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 945 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (ix) FEATURE:

 - (A) NAME/KEY: CDS
 (B) LOCATION: 1..945
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- ATG AAA ATA ATT ATT TTT AGA GTG CTA ACT TTT TTC TTT GTT ATC TTT Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe 10
- TCA GTT AAT GTG GTG GCG AAG GAA TTT ACC TTA GAC TTC TCG ACT GCA Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala
- AAG ACG TAT GTA GAT TCG CTG AAT GTC ATT CGC TCT GCA ATA GGT ACT Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr
- CCA TTA CAG ACT ATT TCA TCA GGA GGT ACG TCT TTA CTG ATG ATT GAT Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp 50 60

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AA' Asi	T TT n Le	A T	yr v	TG A al T 00	CA (GA Sly	TT: Phe	r gr • Va	T AA l As l0	n A	lgg lrg	ACA Thr	AA As	T A	sn	GT: Val	L Pl	rr ie	TAT Tyr		336
CG(Arg	TT Ph	T G(e A]	a A	AT T sp P	TT I	CA	CAT	GT Va 12	T AC l Th O	C I	TT he	CCA Pro	GG Gl	y Tì	CA nr 25	ACA Th:	G GC	:G .a	GTT Val		384
ACA Thi	TTO Let	1 2e	T G	GT G. Ly A	AC A sp S	GT er	AGC Ser 135	Ty:	T AC	C A r T	.CG hr	TTA Leu	CA(Gl:	n Ar	g	GTT Val	GC Al	A a	GGG Gly		432
ATC Ile 145	261	CG Ar	T AC	CG GG	гу м	TG et 50	CAG Gln	ATA	A AA' AS	T C	GC rg	CAT His 155	TCC	TI Le	G i	ACT Thr	AC Th	r :	TCT Ser		480
TAT Tyr	Leu	GA As	T TI p Le	A A: u Me	et S	CG er	CAT His	AG7 Ser	GG Gly	y Tl	CC hr 70	TCA Ser	CTC	AC Th	G (TAG Slm	Se :	T :	GTG Gai		528
GCA Ala	AGA Arg	GC Al	G AT a Me 18	r re	CA CO	ig :	TTT Phe	GTI Val	ACT Thi	: Va	rg .	ACA Thr	GCT Ala	GA Gl	u A	ia 90	÷:	٦ : م د	:3T krg		576
TTT Phe	CGG Arg	CAI Gl: 19	1 11	A CA e Gl	G AG	eg (GGA Gly	TTT Phe 200	CGI	Th	r :	ACA Thr	CTG Leu	GA' Asj 20	P A	AT sp	CT: Let	: 5	s . er		624
GGG Gly	CGT Arg 210	TC: Sei	TA'	T GT r Va	A AT	t 1	ACT Thr 215	GCT Ala	GAA Glu	GA As	T.	/al	GAT Asp 220	CT: Let	A T	CA hr	TTO	; A	AC sn		672
TGG Trp 225	GGA Gly	AGC Arg	TTO	G AG	T AG r Se 23	r V	TC al	CTG Leu	CCT Pro	GA As	рŢ	TAT Tyr 235	CAT His	GG# Gly	A C.	AA ln	GAC Asp	S	CT er 40		720
GTT Val	CGT Arg	GT.A Val	GG; Gly	A AG. Arg 24	g Il	T T e S	CT	TTT Phe	GGA Gly	AG Se 25	r I	TT .	AAT Asn	GCA Ala	A.	le.	CTG Leu 255	G(GA Ly		768
AGC Ser	GTG Val	GCA Ala	TTA Leu 260	ı Ile	A CT	G A	AT .sn	TGT Cys	CAT His 265	CA'	T C	AT (Ala	TCG Ser	Aı	g '	GTT Val	G(A)	CC La		816
AGA Arg	ATG Met	GCA Ala 275	TCI Ser	GAT Asi	GA(3 T 1 P	he :	CCT Pro 280	TCT Ser	AT(G T	GT (ys I	CCG Pro	GCA Ala 285	GA As	T.	GGA Gly	Ac Ar	a g		864
GTC Val	CGT Arg 290	GGG Gly	ATT	ACC Thr	CAC His	5 A:	AT A sn I 95	AAA Lys	ATA Ile	TTO	7 T	rp A	TAE qel	TCA Ser	TC Se	C F	ACT	CT Le	G u		912
GGG (Gly) 305	GCA Ala	ATT Ile	CTG Leu	ATG Met	CGC Arc	, A	SA A	ACT	ATT Ile	AGC Ser	Se	GT er 15									945

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 315 amino acids(B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe

Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala 20 25 30

Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr

Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp 50 55 60

Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp 65 70 75 80

Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn 85 90 95

Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr 100 105 110

Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val

Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly 130 135 140

Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser 150 155 160

Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val 165 170 175

Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg 180 185 190

Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser

Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn 210 215 220

Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser 235 230 235 240

Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly 245 250 255

Ser Val Ala Leu Ile Leu Asn Cys His His His Ala Ser Arg Val Ala 260 265 270

Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg 275 280 285

Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu 290 295 300

Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser 305 310 315

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 267 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..267
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met	Lys	Lys	Thr	Leu	TTA Leu	Ile	Ala	GCA Ala	TCG Ser 10	Leu	TCA Ser	TTT Phe	TTT Phe	TCA Ser 15	GCA Ala	48
AGT Ser	GCG Ala	CTG Leu	Ala	Thr	CCT Pro	Asp	Cys	Val	Thr	Gly	Lys	Val	Glu	TAT Tyr	ACA Thr	96

AAA TAT AAT GAT GAC GAT ACC TTT ACA GTT AAA GTG GGT GAT AAA GAA Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu 40

TTA TTT ACC AAC AGA TGG AAT CTT CAG TCT CTT CTC AGT GCG CAA Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Ser Ala Gln 192 55

ATT ACG GGG ATG ACT GTA ACC ATT AAA ACT AAT GCC TGT CAT AAT GGA Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly 240

GGG GGA TTC AGC GAA GTT ATT TTT CGT 267 Gly Gly Phe Ser Glu Val Ile Phe Arg

- (2) INFORMATION FOR SEQ ID NO:4:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 89 amino acids(B) TYPE: amino acid

 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala

Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr

Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu

Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Ser Ala Gln

Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly

Gly Gly Phe Ser Glu Val Ile Phe Arg

(2) INFORMATION FOR SEQ ID NO:5:

(i)	SEQUE	NCE CHARACTERISTICS:
	(A)	LENGTH: 954 base pairs
	(B)	TYPE: nucleic acid
	(C)	STRANDEDNESS: double
	(D)	TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 (B) LOCATION: 1..954
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

		•	,					LIOIN	. SE	עג ע	NO	: 5 :								
AT Me	G AA t Ly 1	G T	GT 'ys	ATA Ile	ישובי	A TT u Ph	T AZ e Ly	A To	eg gr Tp Va	ra c	rg ' eu (TGC Cys	CT(G TI Le	'A C'I u Le	u G	GT ly 15	TTT Phe		4.8
TC Se	T TC r Se	G G	TA al	TCC Ser 20	TÀT	r TC(c co r Ar	G GA g Gl	u Pr	TT AG Ne Th	CG A	ATA Ile	GAC Asp	TT Ph	e Se	G A	CC hr	CAA Gln		96
CA. Gl:	A AG n Se		AT yr 35	GTC Val	TCI Ser	TCC Ser	TT Le	u As	T AG n Se 0	T AT	TA C	GG Arg	ACA Thr	GA Gl	n II	A T(CG er	ACC Thr		144
CC'	r cr Lei 5	- 0.	AA Lu :	CAT His	ATA Ile	TCT Ser	CA Gl: 5	u GT	G AC y Th	C AC	A I	CG er	GTG Val 60	Sei	G GT	T A7	e	AAC AST		192
65	5	- 443		·	Set	70	Pne	e Ala	a va	G GA l As	pΙ	1e 75	Arg	Gly	. re:	i As	Đ.	∵al 60	:	240
- 7 -	011	• ~.	. a. ,	31 Y	85	Asp	HIS	s Let	1 Ar	r cr g Le	u I. O	le	Ile	Glu	Glr	: As 9	n / 5	Asn	2	288
	· -y1	Va	1	100	GIY	Pne	vai	. AST	105		a Ti	nr .	Asn	Thr	Phe 110	Ty	٠,	arg	3	36
	Ser	11	5	ne	Inr	HIS	TIE	120	Val	CCC Pro	o G1	у '	Val	Thr 125	Thr	Va.	i s	er	3	84
ATG Met	ACA Thr 130	1 11	G G r A	AC .sp	AGC Ser	AGT Ser	TAT Tyr 135	ACC	ACT Thr	CTC Lev	G CA	n 2	CGT Arg 140	GTC Val	GCA Ala	GC(Ala	S C	TG eu	4	32
GAA Glu 145	CGT Arg	TC: Se:	C G r G	GA . ly i	Met	CAA Gln 150	ATC Ile	AGT Ser	CGT Arg	CAC His	Se 15	r I	TG Leu	GTT Val	TCA Ser	TCA Ser	T	AT Yr 60	41	во
CTG Leu	GCG Ala	TT	A A M L	et (GAG Glu 165	TTC Phe	AGT Ser	GGT Gly	AAT Asn	ACA Thr 170	Me	G A	CC :	AGA Arg	GAT Asp	GCA Ala 175	T S	CC er	52	28
AGA Arg	GCA Ala	GTT Val	1	TG (eu / BO	CGT Arg	TTT Phe	GTC Val	ACT Thr	GTC Val 185	ACA Thr	GC.	A G a G	AA (lu)	Ala	TTA Leu 190	CGC Arg	T'	rc ne	57	6
AGG Arg	G±11	ATA 11e 195	: G.	AG A	AGA (GAA Glu	TTT Phe	CGT Arg 200	CAG Gln	GCA Ala	CTO	G T	er (SAA Slu 205	ACT Thr	GCT Ala	CC Pr	er Co	62	:4
GTG Val	TAT Tyr 210	ACG Thr	A7 Me	rg A et I	CG (Pro (GGA Gly 215	GAC Asp	GTG Val	GAC Asp	CT(Let	1 T	CT C hr 1 20	TG . Leu .	AAC Asn	TGG Trp	G0	G .y	67	2

CGA Arg 225	Ile	AGC Ser	AAT Asn	GTG Val	CTT Leu 230	Pro	GAG Glu	TAT	CGG Arg	GGA Gly 235	Glu	GAT Asp	GGT	GTC Val	AGA Arg 240	720
GTG Val	GGG Gly	AGA Arg	ATA Ile	TCC Ser 245	TTT Phe	AAT Asn	AAT Asn	ATA Ile	TCA Ser 250	GCG Ala	ATA Ile	CTG Leu	GGG Gly	ACT Thr 255	GTG Val	768
GCC Ala	GTT Val	ATA Ile	CTG Leu 260	AAT Asn	TGC Cys	CAT His	CAT His	CAG Gln 265	GGG Gly	GCG Ala	CGT Arg	TCT Ser	GTT Val 270	CGC Arg	GCC Ala	816
GTG Val	AAT Asn	GAA Glu 275	GAG Glu	AGT Ser	CAA Gln	CCA Pro	GAA Glu 280	TGT Cys	CAG Gln	ATA Ile	ACT Thr	GGC Gly 285	GAC Asp	AGG Arg	CCT Pro	864
GTT Val	ATA Ile 290	AAA Lys	ATA Ile	AAC Asn	AAT Asn	ACA Thr 295	TTA Leu	TGG Trp	GAA Glu	AGŢ Ser	AAT Asn 300	ACA Thr	GCT Ala	GCA Ala	GCG Ala	912
TTT Phe 305	CTG Leu	AAC Asn	AGA Arg	Lys	TCA Ser 310	CAG Gln	TTT Phe	TTA Leu	TAT Tyr	ACA Thr 315	ACG Thr	GGT Gly	AAA Lys			954

- (2) INFORMATION FOR SEQ ID NO:6:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 318 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe
1 5 10

Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln 20 25 30

Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr 35 40 45

Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn 50 55

His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val 65 70 75 80

Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn 90 95

Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg

Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser

Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu 130 135 140

Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr 145 150 155

Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser 165 170 175

Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe 180 185 190

Arg	g Gln	Ile 195	Gln	Arg	Glu	Phe	Arg 200		Ala	Leu	Ser	Glu 205		Ala	Pro	
Va]	1 Tyr 210	Thr	Met	Thr	Pro	Gly 215	Asp	Val	Asp	Leu	Thr 220		Asn	Trp	Gly	
Arc 225	Ile	Ser	Asn	Val	Leu 230	Pro	Glu	Tyr	Arg	Gly 235	Glu	Asp	Gly	Val	Arg 240	
Val	Gly	Arg	Ile	Ser 245	Phe	Asn	Asn	Ile	Ser 250	Ala	Ile	Leu	Gly	Thr 255	Val	
Ala	Val	Ile	Leu 260	Asn	Cys	His	His	Gln 265	Gly	Ala	Arg	Ser	Val 270	Arg	Ala	
Val	Asn	Glu 275	Glu	Ser	Gln	Pro	Glu 280	Cys	Gln	Ile	Thr	Gly 285	Asp	Arg	Pro	
Val	Ile 290	Lys	Ile	Asn	Asn	Thr 295	Leu	Trp	Glu	Ser	Asn 300	Thr	Ala	Ala	Ala	
Phe 305	Leu	Asn	Arg	Lys	Ser 310	Gln	Phe	Leu	Tyr	Thr 315	Thr	Gly	Lys			
(2)	INFO	DRMAI	CION	FOR	SEQ	ID N	10 : 7 :									
	(i)	(A (B (C	l) LE () TY () ST	NGTH PE: RANE	IARAC I: 26 nucl EDNE GY:	7 ba eic SS:	se p acid doub	airs	;							
	(ii)	MOL	ECUL	E TY	PE:	ANG	(gen	omic)							
	(ix)) NA	ME/K	EY: ON:		67									
	(xi)	SEQ	UENC	E DE	SCRI	PTIO	N: S	EQ I	ои Ф	:7:						
ATG Met 1	AAG Lys	AAG . Lys !	ATG '	TTT Phe 5	ATG Met	GCG Ala	GTT ' Val :	TTA Leu	TTT (Phe 1	GCA ' Ala 1	TTA (Leu .	GCT : Ala !	FCT (Ser)	GTT . Val . 15	AAT Asn	48
GCA Ala	ATG Met	GCG (Ala	GCG (Ala 1 20	GAT Asp	TGT (Cys)	GCT Ala :	AAA (Lys (GGT : Gly :	AAA 1 Lys :	ATT (GAG :	TTT :	CC A Ser I 30	AAG '	TAT Tyr	96

AAT GAG GAT GAC ACA TTT ACA GTG AAG GTT GAC GGG AAA GAA TAC TGG Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp

ACC AGT CGC TGG AAT CTG CAA CCG TTA CTG CAA AGT GCT CAG TTG ACA Thr Ser Arg Trp Asn Leu Gln Pro Leu Gln Ser Ala Gln Leu Thr

GGA ATG ACT GTC ACA ATC AAA TCC AGT ACC TGT GAA TCA GGC TCC GGA Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly 65 70 75 80

55

TTT GCT GAA GTG CAG TTT AAT AAT CAC Phe Ala Glu Val Gln Phe Asn Asn Asp

85

192

240

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 89 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn

Ala Met Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr

Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp

Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr

Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly 65 70 75 80

Phe Ala Glu Val Gln Phe Asn Asn Asp 85

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1241 base pairs

 - (B) TYPE: nucleic acid (C) STRANDEDNESS: double (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGAAAATAA	TTATTTTA	AGTGCTAACT	TTTTTCTTT	TTATCTTTT	C AGTTAATGTG	60
GTGGCGAAGG	AATTTACCTT	AGACTTCTCC	ACTGCAAAG	CGTATGTAG	A TTCGCTGAAT	120
GTCATTCGCT	CTGCAATAGG	TACTCCATTA	CAGACTATT	CATCAGGAG	TACGTCTTTA	180
CTGATGATTG	ATAGTGGCTC	AGGGGATAAT	TTGTTTGCAG	TTGATGTCAG	AGGGATAGAT	240
GCAGAGGAAG	GGCGGTTTAA	TAATCTACGG	CTTATTGTTG	AACGAAATAA	TTTATATGTG	300
ACAGGATTTG	TTAACAGGAC	AAATAATGTT	TTTTATCGCT	TTGCTGATTI	TTCACATGTT	360
ACCTTTCCAG	GTACAACAGC	GGTTACATTG	TCTGGTGACA	GTAGCTATAC	CACGTTACAG	420
CGTGTTGCAG	GGATCAGTCG	TACGGGGATG	CAGATAAATC	GCCATTCGTT	GACTACTTCT	480
TATCTGGATT	TAATGTCGCA	TAGTGGAACC	TCACTGACGC	AGTCTGTGGC	AAGAGCGATG	540
TTACGGTTTG	TTACTGTGAC	AGCTGAAGCT	TTACGTTTTC	GGCAAATACA	GAGGGGATTT	600
		CAGTGGGCGT				660
		GTTGAGTAGC				720
		TTTTGGAAGC				720
		TGCATCGCGA				
		AAGAGTCCGT				840
				·····	ATIGIGGAT	900

TCATCCACTC	TGGGGGCAAT	TCTGATGCGC	AGAACTATTA	GCAGTTGAAC	AGGGGGTAAA	960
TAAAGGAGTT	AAGCATGAAA	AAAACATTAT	TAATAGCTGC	ATCGCTTTCA	TTTTTTTCAG	1020
CAAGTGCGCT	GGCGACGCCT.	GATTGTGTAA	CTGGAAAGGT	GGAGTATACA	AAATATAATG	1080
ATGACGATAC	CTTTACAGTT	AAAGTGGGTG	ATAAAGAATT	ATTTACCAAC	AGATGGAATC	1140
TTCAGTCTCT	TCTTCTCAGT	GCGCAAATTA	CGGGGATGAC	TGTAACCATT	AAAACTAATG	1200
CCTGTCATAA	TGGAGGGGGA	TTCAGCGAAG	TTATTTTTCG	т		1241

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1235 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double

 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATGAAGTGT	LATTATTAT A	A ATGGGTACTO	G TGCCTGTTAC	TGGGTTTTTC	TTCGGTATCC	60
TATTCCCGG	G AGTTTACGAT	AGACTTTTC	ACCCAACAAA	GTTATGTCTC	TTCGTTAAAT	120
AGTATACGG.	A CAGAGATATO	GACCCCTCTT	GAACATATAT	CTCAGGGGAC	CACATCGGTG	180
TCTGTTATT	A ACCACACCC	CGGCAGTTAT	TTTGCTGTGG	ATATACGAGG	GCTTGATGTC	240
TATCAGGCG	C GTTTTGACCA	TCTTCGTCTG	ATTATTGAGC	AAAATAATTT	ATATGTGGCA	300
GGGTTCGTT	A ATÁCGGCAAC	AAATACTTTC	TACCGTTTTT	CAGATTTTAC	ACATATATCA	360
GTGCCCGGT	G TGACAACGGT	TTCCATGACA	ACGGACAGCA	GTTATACCAC	TCTGCAACGT	420
GTCGCAGCG	TGGAACGTTC	CGGAATGCAA	ATCAGTCGTC	ACTCACTGGT	TTCATCATAT	480
CTGGCGTTA	A TGGAGTTCAG	TGGTAATACA	ATGACCAGAG	ATGCATCCAG	AGCAGTTCTG	540
CGTTTTGTC	CTGTCACAGC	AGAAGCCTTA	CGCTTCAGGC	AGATACAGAG	AGAATTTCGT	600
CAGGCACTGT	CTGAAACTGC	TCCTGTGTAT	ACGATGACGC	CGGGAGACGT	GGACCTCACT	660
CTGAACTGGG	GGCGAATCAG	CAATGTGCTT	CCGGAGTATC	GGGGAGAGGA	TGGTGTCAGA	720
GTGGGGAGAA	TATCCTTTAA	TAATATATCA	GCGATACTGG	GGACTGTGGC	CGTTATACTG	780
AATTGCCATC	ATCAGGGGGC	GCGTTCTGTT	CGCGCCGTGA	ATGAAGAGAG	TCAACCAGAA	840
TGTCAGATAA	CTGGCGACAG	GCCTGTTATA	AAAATAAACA	ATACATTATG	GGAAAGTAAT	900
ACAGCTGCAG	CGTTTCTGAA	CAGAAAGTCA	CAGTTTTTAT	ATACAACGGG	TAAATAAAGG	960
AGTTAAGCAT	GAAGAAGATG	TTTATGGCGG	TTTTATTTGC	ATTAGCTTCT	GTTAATGCAA	1020
TGGCGGCGGA	TTGTGCTAAA	GGTAAAATTG	AGTTTTCCAA	GTATAATGAG	GATGACACAT	1080
TTACAGTGAA	GGTTGACGGG	AAAGAATACT	GGACCAGTCG	CTGGAATCTG	CAACCGTTAC	1140
TGCAAAGTGC	TCAGTTGACA	GGAATGACTG	TCACAATCAA	ATCCAGTACC '	TGTGAATCAG	1200
GCTCCGGATT	TGCTGAAGTG	CAGTTTAATA	ATGAC			1235
	•					

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 8 amino acids

(B) TYPE: amino acid(C) STRANDEDNESS: unknown(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: peptide	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:	
Leu Glu His His His His His 1 5	
(2) INFORMATION FOR SEQ ID NO:12:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 29 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:	
GCCATATGAA AATAATTATT TTTAGAGTG	29
(2) INFORMATION FOR SEQ ID NO:13:	
 (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 29 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear 	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:	
GGCTCGAGAC TGCTAATAGT TCTGCGCAT	29
(2) INFORMATION FOR SEQ ID NO:14:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 28 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:	
GCCATATGAA AAAAACATTA TTAATAGC	28
(2) INFORMATION FOR SEQ ID NO:15:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 29 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:	
GGCTCGAGAC GAAAAATAAC TTCGCTGAA	29
(2) INFORMATION FOR SEQ ID NO:16:	
(i) SEQUENCE CHARACTERISTICS:	

(A) LENGTH: 29 base pairs(B) TYPE: nucleic acid(C) STRANDEDNESS: single(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:	
GCCATATGAA GTGTATATTA TTTAAATGG	29
(2) INFORMATION FOR SEQ ID NO:17:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 30 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:	
GGCTCGAGTT TACCCGTTGT ATATAAAAAC	30
(2) INFORMATION FOR SEQ ID NO:16:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 26 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:	
CGCATATGAA GAAGATGTTT ATGGCG	26
(2) INFORMATION FOR SEQ ID NO:19:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 29 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:	
GGCTCGAGGT CATTATTAAA CTGCACTTC	29
(2) INFORMATION FOR SEQ ID NO:20:	
(i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 969 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: DNA (genomic)	
(ix) FEATURE: (A) NAME/KEY: CDS (B) LOCATION: 1969	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:	
ATG AAA ATA ATT TTT AGA GTG CTA ACT TTT TTC TTT GTT ATC TTT	48

Met 1	Lys	; Ile	≥ Ile	≘ Il	e Ph 5	e Ar	g Va	l Le		r Pi	he P	he I	he V	/al :	Ile 15	Phe		
TCA Ser	GTT Val	AAT Asn	GT(Val 20	L va	G GC	G AA	G GA s Gl	u Ph	T AC ie Th	C TI	ra g eu a	AC I sp P	TC I	CG / er 1	ACT Thr	GCA Ala		96
AAG Lys	ACG Thr	TAT Tyr 35	val	A GA' . Asj	T TCO P Ser	G CTO	G AA u As: 4	n Va	C AT	T CG e Ar	C To	er A	CA A la I 45	TA G	GT 1 _Y	ACT Thr		144
CCA Pro	TTA Leu 50	CAG Gln	ACT Thr	AT:	r TCA e Ser	TCA Ser 55	Gl	A GG y Gl	T AC y Th	G TC r Se	r Le	ra c eu L 50	TG A	TG A et I	TT le	GAT Asp		192
AGT Ser 65	GGC Gly	TCA Ser	GGG Gly	GA1 Asp	AAT Asn 70	Leu	TT:	r GCZ e Ala	A GT a Vai	T GA l As	p Va	C Ad	GA GO	GG A ly I	TA le	GAT Asp 80	:	240
GCA Ala	GAG Glu	GAA Glu	GGG Gly	CGG Arg 85	Pne	AAT Asn	AA7	CTA Lev	A CGC Arg 90	g Le	T AT	T GI e Va	TT GA	u A	GA rg 95	AAT Asn	2	288
AAT (Asn)	TTA Leu	TAT Tyr	GTG Val 100	ACA Thr	GGA Gly	TTT Phe	GTT Val	AAC Asn 105	Arg	ACI Thi	AA A As	T AA n As	T GT n Va 11	1 P	ie '	TAT Tyr	3	36
CGC : Arg 1	Prie	GCT Ala 115	GAT Asp	TTT Phe	TCA Ser	CAT His	GTT Val 120	Thr	TTT Phe	CCA Pro	A GG	T AC y Th 12	r Th	A GO	:G (STT Val	3	84
ACA Thr I	rrg Leu 130	TCT Ser	GGT Gly	GAC Asp	AGT Ser	AGC Ser 135	TAT Tyr	ACC Thr	ACG Thr	TTA Leu	CAC Gli	a Ar	T GT g Va	T GC 1 Al	a C	GGG Gly	4	32
ATC A Ile S 145	AGT (Ser)	CGT Arg	ACG Thr	GGG Gly	ATG Met 150	CAG Gln	ATA Ile	AAT Asn	CGC Arg	CAT His 155	Ser	TTC	S ACT	T AC	r s	er 60	4 1	80
TAT C Tyr L	TG (Leu)	GAT ' Asp :	Leu	ATG Met 165	TCG Ser	CAT His	AGT Ser	GGA Gly	ACC Thr 170	TCA Ser	CTG	ACC Thi	G CAC	3 TC 2 Se: 17:	r v	TG al	52	28
GCA A Ala A	GA (ala i	ATG Met :	TTA Leu	CGG Arg	TTT Phe	GTT Val	ACT Thr 185	GTG Val	ACA Thr	GCT Ala	GAZ Glu	GCT Ala 190	Let	A C	GT rg	57	'6
TTT C	rg G	CAA A Sln I .95	rie (GLD	AGG Arg	Gly	Phe	Arg	Thr	Thr	Leu	GAT Asp 205	Asp	CTO Lev	A S	GT er	62	4
GGG CG Gly A: 2:	GT T rg S 10	er 1	'Yr \	Jal	Met '	ACT (Thr : 215	GCT Ala	GAA Glu	GAT Asp	GTT Val	GAT Asp 220	CTT Leu	ACA Thr	TTC Leu	A) As	AC sn	67	2
TGG GG Trp GJ 225	GA A ly A	.GG T .rg L	TG A	ser :	AGC (Ser 1 230	GTC (/al]	CTG Leu	CCT Pro	Asp	TAT Tyr 235	CAT His	GGA Gly	CAA Gln	GAC Asp	TC Se 24	er	72	0
GTT CO Val Ar	GT G cg V	TA G al G	ly A	GA A	ATT T	CT T Ser E	TTT (Phe (Gly .	AGC Ser 250	ATT Ile	AAT Asn	GCA Ala	ATT Ile	CTG Leu 255	GG	SA Y	768	3
AGC GT Ser Va	rg go	la L	TA A eu I 60	TA (le I	CTG A Leu A	AT I	ys I	CAT (His 1 265	CAT His I	CAT His	GCA Ala	TCG Ser	CGA Arg 270	GTT Val	GC Al	C a	816	;
AGA AT Arg Me	E A.	CA TO la Se 75	CT G er A	AT G sp G	SAG I	he P	CT T Pro S	CT : Ser :	ATG 1	IGT (Cys :	CCG Pro	GCA Ala 285	GAT Asp	GGA Gly	AG Ar	A 9	864	

GTC Val	CGT Arg 290	GGG Gly	ATT Ile	ACG Thr	CAC His	AAT Asn 295	AAA Lys	ATA Ile	TTG Leu	TGG Trp	GAT Asp 300	TCA Ser	TCC Ser	ACT Thr	CTG Leu	912
GGG Gly 305	GCA Ala	ATT Ile	CTG Leu	ATG Met	CGC Arg 310	AGA Arg	ACT Thr	ATT Ile	AGC Ser	AGT Ser 315	CTC Leu	GAG Glu	CAC His	CAC His	CAC His 320	960
	CAC His															969

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 323 amino acids
 - (B) TYPE: amino acid
 - (D) TCPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met Lys Ile Ile Ile Phe Arg Val Leu Thr Phe Phe Phe Val Ile Phe Ser Val Asn Val Val Ala Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn 85 90 95 Asn Leu Tyr Val Thr Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly 135 Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg 185 Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Cly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly

l Al	Val	Arg 270	Ser	Ala	His	His	His 265	Cys	Asn	Leu	Ile	Leu 260	Ala	Val	ser
_	Va.		ser	AIA	nis	піз		Cys	ASII	200		260			

Arg Met Ala Ser Asp Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg 280

Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu

Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser Leu Glu His His 305 310 315

His His His

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 294 base pairs
 (B) TYPE: nucleic acid

 - (C) STRANDEDNESS: single
 - (D) TCPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..294
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATG Met 1	AAA Lys	AAA Lys	ACA Thr	TTA Leu 5	Leu	ATA Ile	GCT Ala	GCA Ala	TCG Ser	Leu	TCA Ser	TTT	TTT Phe	TCA Ser	GCA Ala	48
AGT Ser	GCG Ala	CTG Leu	GCG Ala 20	ACG Thr	CCT Pro	GAT Asp	TGT Cys	GTA Val 25	ACT Thr	GGA Gly	AAG Lys	GTG Val	GAG Glu 30	TAT Tyr	ACA Thr	96
AAA Lys	TAT Tyr	AAT Asn 35	GAT Asp	GAC Asp	GAT Asp	ACC Thr	TTT Phe 40	ACA Thr	GTT Val	AAA Lys	GTG Val	GGT Gly 45	GAT Asp	AAA Lys	GAA Glu	144
TTA Leu	TTT Phe 50	ACC Thr	AAC Asn	AGA Arg	TGG Trp	AAT Asn 55	CTT Leu	CAG Gln	TCT Ser	CTT Leu	CTT Leu 60	CTC Leu	AGT Ser	GCG Ala	CAA Gln	192
ATT Ile 65	ACG Thr	GGG Gly	ATG Met	ACT Thr	GTA Val 70	ACC Thr	ATT Ile	AAA Lys	ACT Thr	AAT Asn 75	GCC Ala	TGT Cys	CAT His	AAT Asn	GGA Gly 80	240
GGG Gly	GGA Gly	TTC Phe	AGC Ser	GAA Glu 85	GTT Val	ATT Ile	TTT Phe	CGT Arg	CTC Leu 90	GAG Glu	CAC His	CAC His	CAC His	CAC His 95	CAC His	288
CAC	TG															294

- (2) INFORMATION FOR SEQ ID NO:23:
 - (i) SEQUENCE CHAPACTERISTICS:
 - (A) LENGTH: 97 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Lys Lys Thr Leu Leu Ile Ala Ala Ser Leu Ser Phe Phe Ser Ala

10 15 Ser Ala Leu Ala Thr Pro Asp Cys Val Thr Gly Lys Val Glu Tyr Thr Lys Tyr Asn Asp Asp Asp Thr Phe Thr Val Lys Val Gly Asp Lys Glu Leu Phe Thr Asn Arg Trp Asn Leu Gln Ser Leu Leu Ser Ala Gln Ile Thr Gly Met Thr Val Thr Ile Lys Thr Asn Ala Cys His Asn Gly Gly Gly Phe Ser Glu Val Ile Phe Arg Leu Glu His His His His 85 90 His (2) INFORMATION FOR SEQ ID NO:24: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 981 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (ix) FEATURE: (A) NAME/KEY: CDS
(B) LOCATION: 1..981 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24: ATG AAG TGT ATA TTA TTT AAA TGG GTA CTG TGC CTG TTE CTG CGT

Met 1	Lys	Cys	Ile	TTA Leu 5	Phe	Lys	TGG	GTA Val	Leu 10	TGC Cys	CTG Leu	Leu	Leu	G1y 15	Pne	48
TCT Ser	TCG Ser	GTA Val	TCC Ser 20	TAT Tyr	TCC Ser	CGG Arg	GAG Glu	TTT Phe 25	ACG Thr	ATA Ile	GAC Asp	TTT Phe	TCG Ser 30	Thr	CAA Gln	96
CAA Gln	AGT Ser	TAT Tyr 35	GTC Val	TCT Ser	TCG Ser	TTA Leu	AAT Asn 40	AGT Ser	ATA Ile	CGG Arg	ACA Thr	GAG Glu 45	ATA Ile	TCG Ser	ACC Thr	144
CCT Pro	CTT Leu 50	GAA Glu	CAT His	ATA Ile	TCT Ser	CAG Gln 55	GGG Gly	ACC Thr	ACA Thr	TCG Ser	GTG Val 60	TCT	GTT Val	ATT Ile	AAC Asn	192
His 65	Thr	His	Gly	Ser	Tyr 70	TTT Phe	Ala	Val	Asp	Ile 75	Arg	Gly	Leu	Asp	Val 80	240
TAT	CAG Gln	GCG Ala	CGT Arg	TTT Phe 85	GAC Asp	CAT His	CTT Leu	CGT Arg	CTG Leu 90	ATT Ile	ATT Ile	GAG Glu	CAA Gln	AAT Asn 95	AAT Asn	288
TTA Leu	TAT Tyr	GTG Val	GCA Ala 100	GGG Gly	TTC Phe	GTT Val	AAT Asn	ACG Thr 105	GCA Ala	ACA Thr	AAT Asn	ACT Thr	TTC Phe 110	TAC Tyr	CGT Arg	336
TTT Phe	TCA Ser	GAT Asp 115	TTT Phe	ACA Thr	CAT His	ATA Ile	TCA Ser 120	GTG Val	CCC Pro	GGT Gly	GTG Val	ACA Thr 125	ACG Thr	GTT Val	TCC Ser	384
Met	ACA Thr 130	ACG Thr	GAC Asp	AGC Ser	AGT Ser	TAT Tyr 135	ACC Thr	ACT Thr	CTG Leu	Gln	CGT Arg 140	GTC Val	GCA Ala	GCG Ala	CTG Leu	432

PCT/US96/04093 WO 96/30043

GAI Glu 14!	a Arg	r TCC g Ser	G GGA Gly	ATO Met	Gln 150	Ile	: AGT : Ser	CGI Arg	CAC His	S Ser 155	Let	G GT	r TC l Sei	A TC.	A TAT r Tyr 160	4	8 (
CT(Let	G GCC 1 Ala	TTA Lev	ATG Met	GAG Glu 165	Phe	AGT Ser	GGT	' AAT ' Asn	ACA Thr 170	Met	ACC Thr	AGA Arg	A GAI Asp	GC: Ala 179	A TCC a Ser	5:	2 8
AGA Arg	A GCA Ala	GTT Val	CTG Leu 180	Arg	TTT Phe	GTC Val	ACT Thr	GTC Val 185	ACA Thr	GCA Ala	GAA Glu	GCC Ala	Leu 190	Arg	TTC Phe	57	76
Arg	Gln	1)e	Gln	Arg	Glu	Phe	Arg 200	Gln	Ala	Leu	Ser	Glu 205	Thr	Ala	Pro	62	4:4
Val	Tyr 210	Thr	Met	Thr	Pro	Gly 215	Asp	Val	Asp	Leu	Thr 220	Leu	Asn	Trp	GGG Gly	67	2
CGA Arg 225	ATC Ile	AGC Ser	AAT Asn	GTG Val	CTT Leu 230	CCG Pro	GAG Glu	TAT Tyr	CGG Arg	GGA Gly 235	GAG Glu	GAT Asp	GGT Gly	GTC Val	AGA Arg 240	72	0
GTG Val	GGG Gly	AGA Arg	ATA Ile	TCC Ser 245	TTT Phe	AAT Asn	AAT Asn	ATA Ile	TCA Ser 250	GCG Ala	ATA Ile	CTG Leu	GGG Gly	ACT Thr 255	GTG Val	76	8
Ala	Val	Ile	CTG Leu 260	Asn	Cys	His	His	Gln 265	Gly	Ala	Arg	Ser	Val 270	Arg	Ala	814	5
Val	Asn	Glu 275	GAG Glu	Ser	Gln	Pro	Glu 280	Cys	Gln	Ile	Thr	Gly 285	Asp	Arg	Pro	864	1
Val	Ile 290	Lys	ATA Ile	Asn	Asn	Thr 295	Leu	Trp	Glu	Ser	Asn 300	Thr	Ala	Ala	Ala	912	2
TTT Phe 305	CTG Leu	AAC Asn	AGA Arg	Lys	TCA Ser 310	CAG Gln	TTT Phe	TTA Leu	Tyr	ACA Thr 315	ACG Thr	GGT Gly	AAA Lys	CTC Leu	GAG Glu 320	960)
			CAC His			TG										981	

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 326 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Met Lys Cys Ile Leu Phe Lys Trp Val Leu Cys Leu Leu Leu Gly Phe

Ser Ser Val Ser Tyr Ser Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln

Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr 35 40 45

Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn

His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val 65 70 75 80

Tyr Gln Ala Arg Phe Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn 85 90 95

Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg

Fhe Ser Asp Phe Thr His Ile Ser Val Prc Gly Val Thr Thr Val Ser

Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu 130 135 140

Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Scr Ser Tyr 145 150 155 160

Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser 165 170 175

Arg Ala Val Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe 180 185 190

Arg Gln Ile Gln Arg Glu Phe Arg Gln Ala Leu Ser Glu Thr Ala Pro 195 200 205

Val Tyr Thr Met Thr Pro Gly Asp Val Asp Leu Thr Leu Asn Trp Gly 210 215 220

Arg Ile Ser Asn Val Leu Pro Glu Tyr Arg Gly Glu Asp Gly Val Arg 225 230 235 240

Val Gly Arg Ile Ser Phe Asn Asn Ile Ser Ala Ile Leu Gly Thr Val 245 250 255

Ala Val Ile Leu Asn Cys His His Gln Gly Ala Arg Ser Val Arg Ala 260 265 270

Val Asn Glu Glu Ser Gln Pro Glu Cys Gln Ile Thr Gly Asp Arg Pro 275 280 285

Val Ile Lys Ile Asn Asn Thr Leu Trp Glu Ser Asn Thr Ala Ala Ala 290 295 300

Phe Leu Asn Arg Lys Ser Gln Phe Leu Tyr Thr Thr Gly Lys Leu Glu 305 310 315

His His His His His His 325

(2) INFORMATION FOR SEC ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 294 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..294
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATG AAG AAG ATG TTT ATG GCG GTT TTA TTT GCA TTA GCT TCT GTT AAT Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn 1 5 10

4 R

GCA Ala	ATG Met	GCG Ala	GCG Ala 20	Asp	TGT	GCT Ala	AAA Lys	GGT Gly 25	AAA Lys	ATT	GAG Glu	TTT Phe	TCC Ser 30	AAG Lys	TAT Tyr	96
AAT Asn	GAG Glu	GAT Asp 35	GAC Asp	ACA Thr	TTT Phe	ACA Thr	GTG Val 40	AAG Lys	GTT Val	GAC Asp	GGG Gly	AAA Lys 45	GAA Glu	TAC Tyr	TGG Trp	144
ACC Thr	AGT Ser 50	CGC Arg	TGG Trp	AAT Asn	CTG Leu	CAA Gln 55	CCG Pro	TTA Leu	CTG Leu	CAA Gln	AGT Ser 60	GCT Ala	CAG Gln	TTG Leu	ACA Thr	192
GGA Gly 65	ATG Met	ACT Thr	GTC Val	ACA Thr	ATC Ile 70	AAA Lys	TCC Ser	AGT Ser	ACC Thr	TGT Cys 75	GAA Glu	TCA Ser	GGC Gly	TCC Ser	GGA Gly 80	240
TTT Phe	GCT	GAA Glu	GTG Val	CAG Gln 85	TTT Phe	AAT Asn	AAT Asn	GAC Asp	CTC Leu 90	GAG Glu	CAC His	CAC His	CAC His	CAC His 95	CAC His	288
CAC His	TG															294

- (2) INFORMATION FOR SEQ ID NO:27:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 97 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Met Lys Lys Met Phe Met Ala Val Leu Phe Ala Leu Ala Ser Val Asn

Ala Met Ala Ala Asp Cys Ala Lys Gly Lys Ile Glu Phe Ser Lys Tyr 20 25 30

Asn Glu Asp Asp Thr Phe Thr Val Lys Val Asp Gly Lys Glu Tyr Trp

Thr Ser Arg Trp Asn Leu Gln Pro Leu Leu Gln Ser Ala Gln Leu Thr

Gly Met Thr Val Thr Ile Lys Ser Ser Thr Cys Glu Ser Gly Ser Gly

Phe Ala Glu Val Gln Phe Asn Asn Asp Leu Glu His His His His

- 66 -

His

- (2) INFORMATION FOR SEQ ID NO:28:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs(B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA (genomic)
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGGAATTCAA GGAATTTACC TTAGACTTCT CG

(2) INFORMATION FOR SEQ ID NO:29:

```
(i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 28 base pairs (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear
        (ii) MOLECULE TYPE: DNA (genomic)
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:
   GGCTCGAGTC AACTGCTAAT AGTTCTGC
    (2) INFORMATION FOR SEQ ID NO:30:
                                                                                      2.8
         (i) SEQUENCE CHARACTERISTICS:
               (A) LENGTH: 32 base pairs
               (B) TYPE: nucleic acid
               (C) STRANDEDNESS: single (D) TOPOLOGY: linear
        (ii) MOLECULE TYPE: DNA (genomic)
       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:
  CGGAATTCCG GGAGTTTACG ATAGACTTTT CG
   (2) INFORMATION FOR SEQ ID NO:31:
                                                                                     32
        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 29 base pairs
              (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
              (D) TOPOLOGY: linear
       (ii) MOLECULE TYPE: DNA (genomic)
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:
 GGCTCGAGTT ATTTACCCGT TGTATATAA
  (2) INFORMATION FOR SEQ ID NO:32:
                                                                                    29
       (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 2127 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear
      (ii) MCLECULE TYPE: DNA (genomic)
      (ix) FEATURE:
            (A) NAME/KEY: CDS
             (B) LOCATION: 1..2127
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:
ATG AAA ATA AAA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA TTA ACG
Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr
                                                                                  48
ACG ATG ATG TTT TCC GCC TCG GCT CTC GCC AAA ATC GAA GAA GGT AAA
Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys
CTG GTA ATC TGG ATT AAC GGC GAT AAA GGC TAT AAC GGT CTC GCT GAA Leu Val lle Trp lle Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu
GTC GGT AAG AAA TTC GAG AAA GAT ACC GGA ATT AAA GTC ACC GTT GAG
Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu
                                                                                192
```

5	0				55					60					
CAT CC His Pr 65	G GAT o Asp	AAA Lys	CTG Leu	GAA Glu 70	GAG Glu	AAA Lys	TTC Phe	CCA Pro	CAG Gln 75	GTT Val	GCG Ala	GCA Ala	ACT Thr	80 GGC	240
GAT GG Asp Gl	C CCT y Pro	GAC Asp	ATT Ile 85	ATC Ile	TTC Phe	TGG Trp	GCA Ala	CAC His 90	GAC Asp	CGC Arg	TTT Phe	GGT Gly	GGC Gly 95	TAC Tyr	288
GCT CA Ala Gl	A TCT n Ser	GGC Gly 100	CTG Leu	TTG Leu	GCT Ala	GAA Glu	ATC Ile 105	ACC Thr	CCG Pro	GAC Asp	AAA Lys	GCG Ala 110	TTC Phe	CAG Gln	336
GAC AA Asp Ly	G CTG s Leu 115	Tyr	CCG Pro	TTT Phe	ACC Thr	TGG Trp 120	GAT Asp	GCC Ala	GTA Val	CGT Arg	TAC Tyr 125	AAC Asn	GGC Gly	AAG Lys	384
CTG AT Leu Il 13	e Ala	TAC Tyr	CCG Pro	ATC Ile	GCT Ala 135	GTT Val	GAA Glu	GCG Ala	TTA Leu	TCG Ser 140	CTG Leu	ATT Ile	TAT Tyr	AAC Asn	432
AAA GA Lys As 145	r crg p Leu	CTG Leu	CCG Pro	AAC Asn 150	CCG Pro	CCA Pro	AAA Lys	ACC Thr	TGG Trp 155	GAA Glu	GAG Glu	ATC Ile	CCG Pro	GCG Ala 160	480
CTG GA Leu As	I AAA p Lys	GAA Glu	CTG Leu 165	AAA Lys	GCG Ala	AAA Lys	GGT Gly	AAG Lys 170	AGC Ser	GCG Ala	CTG Leu	ATG Met	TTC Phe 175	AAC Asn	526
CTG CA Leu Gl	n Glu	CCG Pro 180	TAC Tyr	TTC Phe	ACC Thr	TGG Trp	CCG Pro 185	CTG Leu	ATT Ile	GCT Ala	GCT Ala	GAC Asp 190	GGG Gly	GGT Gly	576
TAT GC Tyr Al	G TTC a Phe 195	AAG Lys	TAT Tyr	GAA Glu	AAC Asn	GGC Gly 200	AAG Lys	TAC Tyr	GAC Asp	ATT Ile	AAA Lys 205	GAC Asp	GTG Val	GGC	624
GTG GA Val As 21	p Asn O	Ala	Gly	Ala	Lys 215	Ala	Gly	Leu	Thr	Phe 220	Leu	Val	Asp	Leu	672
ATT AA Ile Ly 225	s Asn	Lys	His	Met 230	Asn	Ala	Asp	Thr	Asp 235	Tyr	Ser	Ile	Ala	Glu 240	720
GCT GC Ala Al	C TTT a Phe	AAT Asn	AAA Lys 245	GGC Gly	GAA Glu	ACA Thr	GCG Ala	ATG Met 250	ACC Thr	ATC Ile	AAC Asn	GGC Gly	CCG Pro 255	TGG Trp	768
GCA TG Ala Tr	G TCC p Ser	AAC Asn 260	ATC Ile	GAC Asp	ACC Thr	AGC Ser	AAA Lys 265	GTG Val	AAT Asn	TAT Tyr	GGT Gly	GTA Val 270	ACG Thr	GTA Val	816
CTG CC Leu Pr	o Thr 275	Phe	Lys	Gly	Gln	Pro 280	Ser	Lys	Pro	Phe	Val 285	Gly	Val	Leu	864
AGC GC Ser Al 29	a Gly	ATT Ile	AAC Asn	GCC Ala	GCC Ala 295	AGT Ser	CCG Pro	AAC Asn	AAA Lys	GAG Glu 300	CTG Leu	GCG Ala	AAA Lys	GAG Glu	912

TTC CTC GAA AAC TAT CTG CTG ACT GAT GAA GGT CTG GAA GCG GTT AAT Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn 305 310 320	960
AAA GAC AAA CCG CTG GGT GCC GTA GCG CTG AAG TCT TAC GAG GAA GAG Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu 325 330 335	1008
TTG GCG AAA GAT CCA CGT ATT GCC GCC ACC ATG GAA AAC GCC CAG AAA Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys 340 345	105€
GGT GAA ATC ATG CCG AAC ATC CCG CAG ATG TCC GCT TTC TGG TAT GCC Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala 355	1104
GTG CGT ACT GCG GTG ATC AAC GCC GCC AGC GGT CGT CAG ACT GTC GAT Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp 370 375 380	1152
GAA GCC CTG AAA GAC GCG CAG ACT TCG AGC TCG AAC AAC AAC AAT Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn Asn 390 395 400	1200
AAC AAT AAC AAC CTC GGG ATC GAG GGA AGG ATT TCA GAA TTC AAG Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Lys 405 410	1248
GAA TTT ACC TTA GAC TTC TCG ACT GCA AAG ACG TAT GTA GAT TCG CTC Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu 420 425 430	1296
AAT GTC ATT CGC TCT GCA ATA GGT ACT CCA TTA CAG ACT ATT TCA TCA Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser 435 440 445	1344
GGA GGT ACG TCT TTA CTG ATG ATT GAT AGT GGC TCA GGG GAT AAT TTG Gly Gly Thr Ser Leu Leu Met 11e Asp Ser Gly Ser Gly Asp Asn Leu 450 460	1392
TTT GCA GTT GAT GTC AGA GGG ATA GAT GCA GAG GAA GGG CGG TTT AAT Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg Phe Asn 470 480	1440
AAT CTA CGG CTT ATT GTT GAA CGA AAT AAT TTA TAT GTG ACA GGA TTT Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr Gly Phe 485 490 495	1488
GTT AAC AGG ACA AAT AAT GTT TTT TAT CGC TTT GCT GAT TTT TCA CAT Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His 500 505 510	1536
GTT ACC TTT CCA GGT ACA ACA GCG GTT ACA TTG TCT GGT GAC AGT AGC Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser 525	1584
TAT ACC ACG TTA CAG CGT GTT GCA GGG ATC AGT CGT ACG GGG ATG CAG Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln 530 540	1632
ATA AAT CGC CAT TCG TTG ACT ACT TCT TAT CTG GAT TTA ATG TCG CAT lle Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His 550 555 560	1680
AGT GGA ACC TCA CTG ACG CAG TCT GTG GCA AGA GCG ATG TTA CGG TTT Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe 565 570 575	1728
GTT ACT GTG ACA GCT GAA GCT TTA CGT TTT CGG CAA ATA CAG AGG GGA Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly	1776

PCT/US96/04093 WO 96/30043

	580			585			590		
						TCT Ser			1824
						AGG Arg 620			1872
						GTA Val			1920
						GCA Ala			1968
						GCA Ala			2016
						GGG			2064
						ATT Ile 700			2112
ATT Ile		TG							2127

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 708 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (Mi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr

Thi Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys

Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu

Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu 50 55 60

His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly

Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr 85 90 95

Ala Gln Ser Gly Leu Leu Ala Glu Ile Thr Pro Asp Lys Ala Phe Gin

Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys

Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn

	13	0					135					140				
Ly: 14!	s As 5	j L	eu]	Leu	Pro	Asn 150	Pro	Pro	Lys	Thr	Trp 155		Glu	lle	Pro Al	la 50
Le	u As	יַל פ	ys (3lu :	Leu 165	Lys	Ala	Lys	GJŻ	Lys 170	Ser	Ala	Leu		Phe As	in
									-05					Asp (Sly Gl	
													205		/al Gl	
											•	220			sp Le	
											233				la Glu 240	`
									•	250				2	ro Trp	>
								•					2	70	hr Val	
							_					28	35		al Leu	
							-				اد	00			′s Glu	
										د	7.5				1 Asn 320	
									د	30				33	u Glu 5	
													35	0	n Lys	
•											er Al	36	5			
							-				ly Ar 38	U				
										23			_		400	
Asn A									4.7	· U				415		
Glu p									_				430	1		
Asn v								_				445				
Gly G											460	,				
Phe A 465										4/:	5				480	
Asn L									770	,				495		
Val A	sn A	rg	Thr 500	Asn	Asn	Val	Phe	Ty: 505	Arg	Phe	Ala	Asp	Phe 510	Ser	His	

יחרווני יאיר בפפחתופאז וא.

Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp Ser Ser 520 Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln 535 Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His 545 550 555 Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr 600 Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile Leu Asn 650 Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe 665 Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met Arg Arg Thr Ile Ser Ser 705 (2) INFORMATION FOR SEQ ID NO:34: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 2136 base pairs(B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (ix) FEATURE: (A) NAME/KEY: CDS (B) LOCATION: 1..2136 (xi) SEQUENCE DESCRIPTION: SEQ ID:NO:34: ATG AAA ATA AAA ACA GGT GCA CGC ATC CTC GCA TTA TCC GCA TTA ACG 48 Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr ACG ATG ATG TIT TCC GCC TCG GCT CTC GCC AAA ATC GAA GAA GGT AAA 96 Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys CTG GTA ATC TGG ATT AAC GGC GAT AAA GGC TAT AAC GGT CTC GCT GAA Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu

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GTC GGT AAG AAA TTC GAG AAA GAT ACC GGA ATT AAA GTC ACC GTT GAG

Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu

CA Hi 6	T CC s Pr 5	G G	AT A sp L	AA ys :	CTG Leu	GAZ Glu 70	4 61	G AA u Ly	AA T /S P	TC he	CCI Pro	2 G 1	AG G ln V	TT al	GC Al	G G a A	CA la	AC' Thi	r Gl	SC Y 0	240
GA As	T GG p Gl	y P:	CT G	AC :	ATT Lle 85	ATO	TTe Ph	C TO	SG G TP A	CA la	CAC His	AS	C C	GC rg	TT' Ph	T G	GT ly	GG(Gl _y 95	' Ty	C	288
GC'	T CA a Gl	A TO		GC (ly I	TG Leu	TTG Leu	GC: Ala	r GA a Gl	A A1 u II 10	e :	ACC Thr	Pr CC	G G O A	AC sp	AAJ Lys	A G(la :	TTC Fhe	CA: Gl:	G n	336
_	C AA D Ly:	12	. 5				1111	12	O AS	p A	Ala	٧ä	1 A:	rg	Tyr 125	As	sr. (3ly	Lys	5	384
CT(Lev	3 AT: 1 Il:	T GC = Al	T Ti	AC C	ro	ATC Ile	GCT Ala 135	va.	T GA 1 G1	A C u A	GCG Ala	TT	A T(u Se	er	CTG Leu	AI Il	T 1	TAT Tyr	AA(Asr	2	432
145						150		PIC	э цу	S I	nr	155	5 G1	.น (Glu	Il	e P	ro	Ala	l !	480
	GAT Asp	-•		1	65	J, J	A-a	D) s	o GI	y L 1	70	ser	. AI	a I	Leu	Me	t P 1	iie 75	Asn		528
CTG Leu	Gln	GA.	A CC u Pr 18	- 1	AC :	rrc Phe	ACC Thr	TGC	Pro 18	эr	TG eu	ATT Ile	GC Al	T (CT la	GAG Asi	o G	GG ly	GGT Gly		576
TAT Tyr	GCG Ala	Pho 19	ر ب	G T; s Ty	AT C	SAA Slu	AAC Asn	GGC Gly 200	LAS	S T	ac yr	GAC Asp	AT Il	e I	AA ys :05	GA(Asp	C G'	rg al	GGC Gly		624
GTG Val	GAT Asp 210		GC Al	T GO a Gl	SC G	La	AAA Lys 215	GCG Ala	GG1	C C	TG eu	ACC Thr	TTO Pho 220	e L	TG	GTI Val	G G	AC sp :	CTG Leu		672
225	AAA Lys		. ريد	- n_	2	30	ASN	AIA	Asp	Tr	ar .	Asp 235	ТУ1	S	er	Ile	Al	a (3lu 240		720
GCT Ala	GCC Ala	TTT Phe	AA: Asi	F AA 1 Ly 24	3 G	GC (GAA Glu	ACA Thr	GCG Ala	A1 M∈ 25	3 T	ACC Thr	ATC Ile	A A	AC sn	GGC Gly	CC Pr 25	0 7	rgg		768
GCA Ala		561	260)	t A	sp.	inr	ser	Lys 265	۷ā	il I	Asn	Tyr	G]	ly :	Val 270	Th	r V	/al		816
CTG Leu		275		. <u>-</u>	5 G ,	ıy (3 11 1	280	ser	ГУ	'S £	ro	Phe	V 2 5	11 (25	Sly	Va.	l L	eu		864
AGC Ser	GCA Ala 290	GGT Gly	ATT	AA(Asi	C GO n Al	la A	SCC A Ala :	AGT Ser	CCG Pro	AA As	C A	JAA .ys	GAG Glu 300	CI	G G	GCG Ala	AAI Lys	4 G 5 G	AG lu		912
TTC Phe 305	CTC Leu	GAA Glu	AAC Asn	TA: Ty:	r Ci	iu L	TG i	ACT Thr	GAT Asp	GA.	u G	GT ly 15	CTG Leu	GA G1	A G	SCG Ala	GT: Val	LA	AT SD 20		960
AAA Lys .	GAC Asp	AAA Lys	CCG Pro	CTC Let 325	וט נ	ST G .y A	cc (la V	STA Val	GCG Ala	CT(Lev	L L	AG ys .	TCT Ser	TA Ty	c G	AG lu	GAZ Glu 335	G	NG lu		1008
TTG (GCG Ala	AAA Lys	GAT Asp	CCP Pro	A CC Ar	T A	TT (SCC Ala	GCC Ala	AC(Thi	C A	TG (GAA Glu	AA As:	C G	CC la	CAG Gln	. Al	A.A Ys		1056

	340		345		350	
Gry Gru	ATC ATG CC lle Met Pro	G AAC ATC o Asn Ile	CCG CAG AT Pro Gln Me 360	et Ser Ala P	TTC TGG TAT GC The Trp Tyr Al	C 1104
GTG CGT A Val Arg T 370	ACT GCG GTG Thr Ala Val	G ATC AAC l Ile Asn 375	GCC GCC AG Ala Ala Se	GC GGT CGT C er Gly Arg G 380	AG ACT GTC GA	T 1152
GAA GCC C Glu Ala I 385	TG AAA GAO eu Lys Asp	GCG CAG Ala Gln 390	ACT TCG AG Thr Ser Se	GC TCG AAC A er Ser Asn A 395	AC AAC AAC AA' sn Asn Asn As 400	n
AAC AAT A Asn Asn A	AC AAC AAC sn Asn Asr 405	Leu Gly	ATC GAG GG Ile Glu Gl 41	y Arg Ile S	CA GAA TTC CGG er Glu Fhe Arg - 415	3 1248 9
GAG TTT A Glu Phe T	CG ATA GAC hr Ile Asp 420	TTT TCG Phe Ser	ACC CAA CA Thr Gln Gl 425	A AGT TAT G n Ser Tyr V	TC TCT TCG TT al Ser Ser Let 430	1296
Asn Ser I	TA CGG ACA le Arg Thr 35	Glu Ile	TCG ACC CC Ser Thr Pro 440	o Leu Glu H	AT ATA TCT CAG is Ile Ser Gln 15	1344
GGG ACC A Gly Thr T 450	CA TCG GTG hr Ser Val	TCT GTT Ser Val 455	ATT AAC CA Ile Asn Hi	C ACC CAC GO s Thr His Gl 460	GC AGT TAT TTT Ly Ser Tyr Phe	1392
GCT GTG G Ala Val A: 465	ATA CGA sp lle Arg	GGG CTT Gly Leu 470	GAT GTC TA Asp Val Ty:	T CAG GCG CG r Gln Ala Ar 475	ST TTT GAC CAT TG Phe Asp His 480	
CTT CGT CT Leu Arg Le	TG ATT ATT Bu Ile Ile 485	GAG CAA A	AAT AAT TT Asn Asn Lei 490	u Tyr Val Al	CA GGG TTC GTT a Gly Phe Val 495	1488
AAT ACG GC Asn Thr Al	CA ACA AAT La Thr Asn 500	ACT TTC Thr Phe	TAC CGT TTT Tyr Arg Phe 505	T TCA GAT TT e Ser Asp Ph	T ACA CAT ATA e Thr His Ile 510	1536
TCA GTG CC Ser Val Pr 51	co Gly Val	Thr Thr V	GTT TCC ATO Val Ser Met 520	G ACA ACG GA Thr Thr As 52	C AGC AGT TAT p Ser Ser Tyr 5	1584
ACC ACT CT Thr Thr Le 530	G CAA CGT Gln Arg	GTC GCA C Val Ala A 535	GCG CTG GAA Ala Leu Glu	A CGT TCC GG 1 Arg Ser Gl 540	A ATG CAA ATC y Met Gln Ile	1632
AGT CGT CA Ser Arg Hi 545	C TCA CTG s Ser Leu	GTT TCA 1 Val Ser 5 550	CCA TAT CTG Ser Tyr Leu	G GCG TTA ATO Ala Leu Me 555	G GAG TTC AGT t Glu Phe Ser 560	1680
GGT AAT AC Gly Asn Th	A ATG ACC r Met Thr 565	AGA GAT G Arg Asp A	SCA TCC AGA Ala Ser Arg 570	Ala Val Le	G CGT TTT GTC u Arg Phe Val 575	1728
ACT GTC AC Thr Val Th	A GCA GAA r Ala Glu 580	GCC TTA C Ala Leu A	GC TTC AGG rg Phe Arg 585	CAG ATA CAG Glr Ile Glr	G AGA GAA TTT n Arg Glu Phe 590	1776
CGT CAG GC Arg Gln Al 59	a Leu Ser	Glu Thr A	CT CCT GTG la Pro Val	TAT ACG ATO Tyr Thr Met	G ACG CCG GGA Thr Pro Gly	1824
GAC GTG GA Asp Val As 610	C CTC ACT p Leu Thr	CTG AAC T Leu Asn T 615	GG GGG CGA	ATC AGC AAT 11e Ser Asr 620	GTG CTT CCG	1872

625	•	_	,	010	630	GIY	val	Arg	۷āl	635	Arg	Ile	Ser	Phe	AAT Asn 640	1920
				645	neu	917	1111	VEI	650	Val	Ile	Leu	Asn	Cys 655		1968
CAT His	CAG Gln	GGG	GCG Ala 660	CGT Arg	TCT Ser	GTT Val	CGC Arg	GCC Ala 665	GTG Val	AAT Asn	GAA Glu	GAG Glu	AGT Ser 670	CAA Gln	CCA Prc	2016
GAA Glu	TGT Cys	CAG Gln 675	ATA Ile	ACT Thr	GGC Gly	GAC Asp	AGG Arg 680	CCT Pro	GTT Val	ATA Ile	AAA Lys	ATA Ile 685	AAC Asn	AAT Asn	ACA Thr	2064
TTA Leu	TGG Trp 690	GAA Glu	AGT Ser	AAT Asn	ACA Thr	GCT Ala 695	GCA Ala	GCG Ala	TTT Phe	CTG Leu	AAC Asn 700	AGA Arg	AAG Lys	TCA. Ser	CAG Gln	2112
TTT Phe 705	TTA Leu	TAT Tyr	ACA Thr	ACG Thr	GGT Gly 710	AAA Lys	TA									2136

- (2) INFORMATION FOR SEQ ID NO:35:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 711 amino acids
 (B) TYPE: amino acid

 - (D) TCPOLOGY: linear
 - (ii) MOLECULE TYPE: protein
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Met Lys Ile Lys Thr Gly Ala Arg Ile Leu Ala Leu Ser Ala Leu Thr 10

Thr Met Met Phe Ser Ala Ser Ala Leu Ala Lys Ile Glu Glu Gly Lys

Leu Val Ile Trp Ile Asn Gly Asp Lys Gly Tyr Asn Gly Leu Ala Glu

Val Gly Lys Lys Phe Glu Lys Asp Thr Gly Ile Lys Val Thr Val Glu

His Pro Asp Lys Leu Glu Glu Lys Phe Pro Gln Val Ala Ala Thr Gly

Asp Gly Pro Asp Ile Ile Phe Trp Ala His Asp Arg Phe Gly Gly Tyr

Ala Gin Ser Gly Leu Leu Ala Glu Ile Thr Prc Asp Lys Ala Phe Gin

Asp Lys Leu Tyr Pro Phe Thr Trp Asp Ala Val Arg Tyr Asn Gly Lys

Leu Ile Ala Tyr Pro Ile Ala Val Glu Ala Leu Ser Leu Ile Tyr Asn

Lys Asp Leu Leu Pro Asn Pro Pro Lys Thr Trp Glu Glu Ile Pro Ala

Leu Asp Lys Glu Leu Lys Ala Lys Gly Lys Ser Ala Leu Met Phe Asn

Leu Gln Glu Pro Tyr Phe Thr Trp Pro Leu Ile Ala Ala Asp Gly Gly 180

SECULIO SESONASA IA.

Tyr Ala Phe Lys Tyr Glu Asn Gly Lys Tyr Asp Ile Lys Asp Val Gly Val Asp Asn Ala Gly Ala Lys Ala Gly Leu Thr Phe Leu Val Asp Leu Ile Lys Asn Lys His Met Asn Ala Asp Thr Asp Tyr Ser Ile Ala Glu 235 Ala Ala Phe Asn Lys Gly Glu Thr Ala Met Thr Ile Asn Gly Pro Trp Ala Trp Ser Asn Ile Asp Thr Ser Lys Val Asn Tyr Gly Val Thr Val Leu Pro Thr Phe Lys Gly Gln Pro Ser Lys Pro Phe Val Gly Val Leu 280 Ser Ala Gly Ile Asn Ala Ala Ser Pro Asn Lys Glu Leu Ala Lys Glu 295 Phe Leu Glu Asn Tyr Leu Leu Thr Asp Glu Gly Leu Glu Ala Val Asn Lys Asp Lys Pro Leu Gly Ala Val Ala Leu Lys Ser Tyr Glu Glu Glu Leu Ala Lys Asp Pro Arg Ile Ala Ala Thr Met Glu Asn Ala Gln Lys Gly Glu Ile Met Pro Asn Ile Pro Gln Met Ser Ala Phe Trp Tyr Ala Val Arg Thr Ala Val Ile Asn Ala Ala Ser Gly Arg Gln Thr Val Asp Glu Ala Leu Lys Asp Ala Gln Thr Ser Ser Ser Asn Asn Asn Asn 390 Asn Asn Asn Asn Leu Gly Ile Glu Gly Arg Ile Ser Glu Phe Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe Asp His 475 Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr His Ile Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg Phe Val

				56	5				57	0					57	5	
Th	r Va	l Th	r Al 58	a Gl O	u Al	a Le	u Ar	g Phe 589	e Ar	g Gl	n I	le G		4.rg		: Phe	
Ar	g Gl	n Al 59	a Le	u Se	r Gli	u Thi	r Ala	a Pro	Va.	l Ty	r Tì		et 1	Chr	Pro	Gly	
As	p Va 61	l As	p Le	u Th	r Lei	ASI 615	n Tri	o Gly	Arg	g Il	e Se 62	r A:	sn V	/al	Let	Pro	
Gl: 62	и Ту: 5	r Ar	g Gl	y Glı	1 Ast 630	Gly	/ Val	Arg	Val	1 G1 63	y Ar 5	g I	ie S	er	Phe	Asn 640	
Ası	n Ile	e Sei	r Ala	a Ile 649	E Leu	Gly	' Thr	. Val	Ala 650	a Vai	l Il	e Le	u A	.sn	Cys 655		
His	Gl:	ı Gly	/ Ala	a Arg	; Ser	· Val	Arg	Ala 665	Val	. Ası	n Gl	u Ġl		er 70	Gln	Pro	
Gli	ı Cys	Glr 675	ı Ile	Thi	Gly	Asp	Arg 680	Pro	Val	. Ile	E Ly	s Il 68		sn	Asn	Thr	
Lev	Trp 690	Glu	Ser	Asn	Thr	Ala 695	Ala	Ala	Phe	Lei	70	n Ar	g L	y's	Ser	Gln	
Phe 705	Leu	Tyr	Thr	Thr	Gly 710												
(2)	INF	ORMA	TION	FOR	SEQ	ID '	NO : 3	6 :									
) MO) FE. (.	C) S' D) T LECU: ATUR! A) N	TRAN OPOL LE T E: AME/:	DEDNI OGY : YPE : KEY :		sing ear (gen	gle nomic	:)								
	(xi) SE	QUEN:	CE Di	ESCRI	PTIC	ON: 5	SEQ I	D NO	D:3€	:						
ATG Met 1	AAA Lys	AAG Lys	ACA Thr	GCT Ala 5	ATC Ile	GCG Ala	ATT Ile	GCA Ala	GTG Val 10	GCA Ala	CTG Leu	GCT Ala	GG G1	T I	TC (he 1	GCT Ala	
ACC Thr	GTT Val	GCG Ala	CAA Gln 20	GCT Ala	GAC Asp	TAC Tyr	AAG Lys	GAC ASP .	GAC Asp	GAT Asp	GAC Asp	AAG Lys	AA(Ly:	s L	TT (eu (SAA Slu	
TTC Phe	AAG Lys	GAA Glu 35	TTT Phe	ACC Thr	TTA Leu	GAC Asp	TTC Phe 40	TCG : Ser :	ACT Thr	GCA Ala	AAG Lys	ACG Thr 45	TA: Tyi	r G	TA (SAT Asp	
TCG Ser	CTG Leu 50	AAT Asn	GTC Val	ATT Ile	CGC Arg	TCT Ser 55	GCA Ala	ATA (Ile (GGT Gly	ACT Thr	CCA Pro 60	TTA Leu	CAC Glr	A :	CT A	ATT :le	
TCA Ser 65	TCA Ser	GGA Gly	GGT Gly	ACG Thr	TCT Ser 70	TTA Leu	CTG . Leu .	ATG A Met I	ATT	GAT Asp 75	AGT Ser	GGC Gly	TCA Ser	. G	GG G ly A	AT Sp 80	
AAT Asn	TTG Leu	TTT Phe	GCA Ala	GTT Val E5	GAT Asp	GTC : Val :	AGA Arg	GGG A	TA (le)	GAT Asp	GCA Ala	GAG Glu	GAA Glu	G.	GG C ly A	GG .rg	

336

TTT AAT AAT CTA CGG CTT ATT GTT GAA CGA AAT AAT TTA TAT GTG ACA

SUUCILI SMIU GESUUNSMI IN-

Phe	e Ası	n Ası	let 100	ı Arg	g Lei	ı Ile	≥ Vai	105 105	ı Arg	g As:	n Ası	n Lei	11		1 Thr	
GG! Gly	A TTT	GTT Val	. Asr	AGO Arg	G ACA Th:	A AAT Asi	AAI Asr 120	ı Val	TTT.	r TAS	T CGC	TT: Phe 125	≥ Ala	T GA a As	T TTT p Phe	38
TCA Ser	CAT His	va.	ACC Thr	TTT Phe	CCA Pro	GGT Gly 135	The	A ACA	GCC Ala	GTT Val	T ACA Thr 140	Let	TC:	r GG	T GAC y Asp	43
AGT Ser 145	267	TAT Tyr	ACC Thr	ACC	Leu 150	GIN	CGT Arg	GTT Val	GCA Ala	GGG Gly 155	'Ile	AGT Ser	CGI Arg	r AC	G GGG r Gly 160	48
ATG Met	CAG Gln	ATA Ile	AAT Asn	CGC Arg 165	Hls	TCG Ser	TTG Leu	ACT Thr	ACT Thr 170	Ser	TAT Tyr	CTG Leu	GAT Asp	Let 179	A ATG A Met	528
TCG Ser	CAT His	AGT Ser	GGA Gly 180	Thr	TCA Ser	CTG Leu	ACG Thr	CAG Gln 185	Ser	GTG Val	GCA Ala	AGA Arg	GCG Ala 190	Met	TTA Leu	576
CGG Arg	TTT Phe	GTT Val 195	Thr	GTG Val	ACA Thr	GCT Ala	GAA Glu 200	GCT Ala	TTA Leu	CGT Arg	TTT Phe	CGG Arg 205	CAA Gl::	ATZ IIE	CAG Glm	624
AGG Arg	GGA Gly 210	TTT Phe	CGT Arg	ACA Thr	ACA Thr	CTG Leu 215	GAT Asp	GAT Asp	CTC Leu	AGT Ser	GGG Gly 220	CGT Arg	TCT Ser	TAT	GTA Val	672
225	Inr	Aia	GIU	Asp	Va1 230	Asp	Leu	Thr	Leu	Asn 235	TGG Trp	Gly	Arç	Leu	Ser 240	720
AGC Ser	GTC Val	CTG Leu	CCT Pro	GAC Asp 245	TAT Tyr	CAT His	GGA Gly	CAA Gln	GAC Asp 250	TCT Ser	GTT Val	CGT Arg	GTA Val	GGA Gly 255	AGA Arg	768
ATT Ile	TCT Ser	TTT Phe	GGA Gly 260	AGC Ser	ATT Ile	AAT Asn	GCA Ala	ATT Ile 265	CTG Leu	GGA Gly	AGC Ser	GTG Val	GCA Ala 270	TTA Leu	ATA' Ile	816
CTG Leu	AAT Asn	TGT Cys 275	CAT His	CAT His	CAT His	Ala	TCG Ser 280	CGA Arg	GTT Val	GCC Ala	AGA Arg	ATG Met 285	GCA Ala	TCT Ser	GAT Asp	864
GAG Glu	TTT Phe 290	CCT Pro	TCT Ser	ATG Met	Cys	CCG Pro 295	GCA Ala	GAT Asp	GGA Gly	Arg	GTC Val 300	CGT Arg	GGG Gly	ATT Ile	ACG Thr	912
CAC His 305	AAT Asn	AAA Lys	ATA Ile	Leu	TGG Trp 310	GAT Asp	TCA Ser	TCC . Ser	Thr	CTG Leu 315	GGG Gly	GCA Ala	ATT Ile	CTG Leu	ATG Met 320	960
CGC Arg	AGA Arg	ACT Thr	ATT Ile	AGC Ser 325	AGT Ser	TG										981

- (2) INFORMATION FOR SEQ ID NO:37:

 - (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 326 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Lys Lys Leu Glu Phe Lys Glu Phe Thr Leu Asp Phe Ser Thr Ala Lys Thr Tyr Val Asp Ser Leu Asn Val Ile Arg Ser Ala Ile Gly Thr Pro Leu Gln Thr Ile Ser Ser Gly Gly Thr Ser Leu Leu Met Ile Asp Ser Gly Ser Gly Asp Asn Leu Phe Ala Val Asp Val Arg Gly Ile Asp Ala Glu Glu Gly Arg 90 Phe Asn Asn Leu Arg Leu Ile Val Glu Arg Asn Asn Leu Tyr Val Thr 105 Gly Phe Val Asn Arg Thr Asn Asn Val Phe Tyr Arg Phe Ala Asp Phe Ser His Val Thr Phe Pro Gly Thr Thr Ala Val Thr Leu Ser Gly Asp 135 Ser Ser Tyr Thr Thr Leu Gln Arg Val Ala Gly Ile Ser Arg Thr Gly Met Gln Ile Asn Arg His Ser Leu Thr Thr Ser Tyr Leu Asp Leu Met Ser His Ser Gly Thr Ser Leu Thr Gln Ser Val Ala Arg Ala Met Leu 180 Arg Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln 200 Arg Gly Phe Arg Thr Thr Leu Asp Asp Leu Ser Gly Arg Ser Tyr Val Met Thr Ala Glu Asp Val Asp Leu Thr Leu Asn Trp Gly Arg Leu Ser 230 Ser Val Leu Pro Asp Tyr His Gly Gln Asp Ser Val Arg Val Gly Arg 245 Ile Ser Phe Gly Ser Ile Asn Ala Ile Leu Gly Ser Val Ala Leu Ile 260 265 270 Leu Asn Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp 280 Glu Phe Pro Ser Met Cys Pro Ala Asp Gly Arg Val Arg Gly Ile Thr 295 His Asn Lys Ile Leu Trp Asp Ser Ser Thr Leu Gly Ala Ile Leu Met 310 315 Arg Arg Thr Ile Ser Ser

(2) INFORMATION FOR SEQ ID NO:38:

COCCID- SIMO GESOCIASM IN-

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 990 base pairs

 - (B) TYPE: nucleic acid (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 1..990

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

												٠.					
	ATO Met	- 177.	A AA s Ly	G AC	A GC r Al	T AT a Il 5	C GC e Al	G AT	T GC e Al	A GTO	l Al	A CT a Le	G GC u Al	T GG a Gl	y Fi	TC GCT ne Ala 15	Γ 4ε i
	ACC	GT'	r gc l Al	G CA a Gl 2	n Al	T GA a As	C TA	C AAG	G GAG S Asj 2	p Ası	C GA P As	T GA p As	C AA p Ly	s Ly	G CT S Le	TT GAA	5 S 6
	TTC Phe	CGC Arg	G GA Gl 3	u Pn	T AC e Th	G ATZ	A GA0 ≘ Asl	C TTT P Phe 40	e Sei	ACC Thi	CAir Gli	A CA n Gl:	A AG n Se 4	r Ty	T GT T Va	C TCT	144
	Jei	50)	.ı se.	I 11(e Arg	5 Thi	GII	ı Ile	e Ser	Thi	r Pro	o Le	u Gl	u Hi	T ATA s Ile	
	65	GII	. 61	y In:	rini	7 Sei	val	. Ser	. Vel	. Ile	2 AST	n His	Th:	r Hi	s Gl	C AGT y Ser 80	240
	TAT Tyr	TTT	GCT Ala	r GT(a Val	G GAT Asp 85	: TTE	CGA Arg	GGG Gly	CTI Leu	GAT Asp 90	Val	TAT Tyr	CAC Glr	G GCC	G CG a Ar	T TTT g Phe 5	288
•	qen	nis	ne t	100	Let	ı ile	. Ile	Glu	Gln 105	Asn	Asn	Leu	Туг	7 Va)	l Ala	A GGG B Gly	336
•	- 116	VAI	115	ini	Ala	Thr	Asn	120	Phe	Tyr	hrg	Phe	Ser 125	yeb	Phe	ACA Thr	384
•	•	130	261	val	Pro	GIY	135	Thr	Thr	Val	Ser	Met 140	Thr	Thr	Asr	AGC Ser	432
3	45	1 y -	ilil	1111	Leu	150	Arg	Val	Ala	Ala	Leu 155	Glu	Arg	Ser	Gly	ATG Met 160	480
9	AA Sln	ATC Ile	AGT Ser	CGT Arg	CAC His 165	TCA Ser	CTG Leu	GTT Val	TCA Ser	TCA Ser 170	TAT Tyr	CTG Leu	GCG Ala	TTA Leu	ATG Met 175	GAG Glu	528
Ţ	TC he	AGT Ser	GGT Gly	AAT Asn 180	ACA Thr	ATG Met	ACC Thr	AGA Arg	GAT Asp 185	GCA Ala	TCC Ser	AGA Arg	GCA Ala	GTT Val 190	CTG Leu	CGT Arg	57€
Þ	TT he	GTC Val	ACT Thr 195	GTC Val	ACA Thr	GCA Ala	GAA Glu	GCC Ala 200	TTA Leu	CGC Arg	TTC Phe	AGG Arg	CAG Gln 205	ATA Ile	CAG Gln	AGA Arg	624
G G	T U	TTT Phe 210	CGT Arg	CAG Gln	GCA Ala	CTG Leu	TCT Ser 215	GAA Glu	ACT Thr	GCT Ala	CCT Pro	GTG Val 220	TAT Tyr	ACG Thr	ATG Met	ACG Thr	€72
P	CG (ro (25	GGA Gly	GAC Asp	GTG Val	GAC Asp	CTC Leu 230	ACT Thr	CTG Leu	AAC Asn	Trp	GGG Gly 235	CGA Arg	ATC Ile	AGC Ser	AAT Asn	GTG Val 240	720
L.	TT (eu)	CCG Pro	GAG Glu	TAT Tyr	CGG Arg	GGA Gly	GAG Glu	GAT Asp	GGT Gly	GTC Val	AGA Arg	GTG Val	GGG Gly	AGA Arg	ATA Ile	TCC Ser	768

				245					250					255		
TTT Phe	AAT Asn	AAT Asn	ATA Ile 260	TCA Ser	GCG Ala	ATA Ile	CTG Leu	GGG Gly 265	ACT Thr	GTG Val	GCC Ala	GTT Val	ATA Ile 270	CTG Leu	AAT Asn	816
TGC Cys	CAT His	CAT His 275	CAG Gîn	GGG Gly	GCG Ala	CGT Arg	TCT Ser 280	GTT Val	CGC Arg	GCC Ala	GTG Val	AAT Asn 285	GAA Glu	GAG Glu	AGT Ser	864
CAA Gln	CCA Pro 290	GAA Glu	TGT Cys	CAG Gln	ATA Ile	ACT Thr 295	GGC Gly	GAC Asp	AGG Arg	CCT Pro	GTT Val 300	ATA Ile	AAA Lys	ATA Ile	AAC Asn	912
AAT Asn 305	ACA Thr	TTA Leu	TGG Trp	GAA Glu	AGT Ser 310	AAT Asn	ACA Thr	GCT Ala	GCA Ala	GCG Ala 315	TTT Phe	CTG Leu	AAC Asn	Arg	AAG Lys 320	960
TCA Ser	CAG Gln	TTT Phe	Leu	TAT Tyr 325	ACA Thr	ACG Thr	GGT Gly	AAA Lys	TA 330	-		•			•	990
(2)	TNFO	יר מאום.	TON	EOD	CEO	TD 11										

- INFORMATION FOR SEQ ID NO:39:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 329 amino acids(B) TYPE: amino acid

 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: protein
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Pne Ala Thr Val Ala Gln Ala Asp Tyr Lys Asp Asp Asp Lys Lys Leu Glu

Phe Arg Glu Phe Thr Ile Asp Phe Ser Thr Gln Gln Ser Tyr Val Ser

Ser Leu Asn Ser Ile Arg Thr Glu Ile Ser Thr Pro Leu Glu His Ile

Ser Gln Gly Thr Thr Ser Val Ser Val Ile Asn His Thr His Gly Ser 65 70 75 80

Tyr Phe Ala Val Asp Ile Arg Gly Leu Asp Val Tyr Gln Ala Arg Phe 85 90 95

Asp His Leu Arg Leu Ile Ile Glu Gln Asn Asn Leu Tyr Val Ala Gly 105

Phe Val Asn Thr Ala Thr Asn Thr Phe Tyr Arg Phe Ser Asp Phe Thr

His Ile Ser Val Pro Gly Val Thr Thr Val Ser Met Thr Thr Asp Ser

Ser Tyr Thr Thr Leu Gln Arg Val Ala Ala Leu Glu Arg Ser Gly Met

Gln Ile Ser Arg His Ser Leu Val Ser Ser Tyr Leu Ala Leu Met Glu 170

Phe Ser Gly Asn Thr Met Thr Arg Asp Ala Ser Arg Ala Val Leu Arg 180

Phe Val Thr Val Thr Ala Glu Ala Leu Arg Phe Arg Gln Ile Gln Arg

		195					200					205			
Glu	Phe 210	Arg	Gln	Ala	Leu	Ser 215	Glu	Thr	Ala	Pro	Val 220	Tyr	Thr	Met	Thr
Pro 225	Gly	Asp	Val	Asp	Leu 230	Thr	Leu	Asn	Trp	Gly 235	Arg	Ile	Ser	Asn	Val 240
Leu	Pro	Glu	Tyr	Arg 245	Gly	Glu	Asp	Gly	Val 250	Arg	Val	Gly	Arg	Il∈ 255	Ser
Phe	Asn	Asn	Ile 260	Ser	Ala	Ile	Leu	Gly 265	Thr	Val	Ala	Val	Ile 270	Leu	Asn
Cys	His	His 275	Gln	Gly	Ala	Arg	Ser 280	Val	Arg	Ala	Val	Asn 285	Glu	Glu	Ser
Gln	Pro 290	Glu	Cys	Gln	Ile	Thr 295	Gly	Asp	Arg	Pro	Val 300	Ile	Lys	Ile	Asn
Asn 305	Thr	Leu	Trp	Glu	Ser 310	Asn	Thr	Ala	Ala	Ala 315	Phe	Leu	Asn	Arg	Lys 320
Ser	Gln	Phe	Leu	Tyr 325	Thr	Thr	Gly	Lys							

CLAIMS

What is claimed is:

SDOCID: ~WO

5 1. A method of treatment comprising: a) providing: antitoxin directed against at least a portion of an Escherichia coli i) verotoxin in an aqueous solution in therapeutic amount that is administrable. and 10 ii) an intoxicated subject; and administering said antitoxin to said subject. b) The method of Claim 1 wherein said Escherichia coli verotoxin is recombinant. 2. 15 The method of Claim 1 wherein said antitoxin is an avian antitoxin. 3. The method of Claim 2 wherein said recombinant Escherichia coli verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the Escherichia coli verotoxin VT1 sequence. 20 The method of Claim 2 wherein said recombinant Escherichia coli verotoxin is 5. a fusion protein comprising a non-verotoxin protein sequence and a portion of the Escherichia coli verotoxin VT2. sequence. 25 The method of Claim 1 wherein said subject is an adult. 6. The method of Claim 1 wherein said subject is a child. 7. The method of Claim 1 wherein said administering is parenteral. 8. 30 The method of Claim 1 wherein said administering is oral. 9.

10. A method of prophylactic treatment comprising:

a) providing:

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- i) an antitoxin directed against at least one *Escherichia coli* verotoxin in an aqueous solution in therapeutic amount that is parenterally administrable, and
 - ii) at least one subject is at risk of diarrheal disease; and
- b) parenterally administering said antitoxin to said subject.
- 11. The method of Claim 10, wherein said subject is at risk of developing extraintestinal complications of *Escherichia coli* infection.
 - 12. The method of Claim 11, wherein said extra-intestinal complication is hemolytic uremic syndrome.
- 15 13. A composition comprising neutralizing antitoxin directed against at least one Escherichia coli verotoxin in an aqueous solution in therapeutic amounts.
 - 14. The composition of Claim 13 wherein said *Escherichia coli* verotoxin is a recombinant toxin.
 - 15. The composition of Claim 14 wherein said recombinant *Escherichia coli* verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the *Escherichia coli* verotoxin VT1 sequence.
- 25 16. The composition of Claim 14 wherein said recombinant Escherichia coli verotoxin is a fusion protein comprising a non-verotoxin protein sequence and a portion of the Escherichia coli verotoxin VT2 sequence.
- 17. The composition of Claim 14 wherein said antitoxin is directed against a portion of at least one Escherichia coli verotoxin.
 - 18. The composition of Claim 14 wherein said portion of Escherichia coli is selected from the group consisting of subunit A and subunit B of VT1.

19. The composition of Claim 14 wherein said portion of Escherichia coii is selected from the group consisting of subunit A and subunit B of VT2.

- 20. The composition of Claim 14 wherein said antitoxin is directed against a portion of at least one *Escherichia coli* verotoxin.
 - 21. The composition of Claim 14 wherein said antitoxin is an avian antitoxin.
 - 22. A method of treatment of enteric bacterial infections comprising:
 - a) providing:

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- i) an avian antitoxin directed against at least one verotoxin produced by *Escherichia coli* in an aqueous solution in therapeutic amount that is parenterally administrable, and
 - ii) at least one infected subject; and
- b) parenterally administering said avian antitoxin to said subject.
- 23. The method of Claim 18 wherein said *Escherichia coli* is selected from the group consisting of *Escherichia coli* serotypes O157:H7. O1:NM; O2:H5; O2:H7: O4:NM: O4:H10: O5:NM; O5:H16: O6:H1; O18:NM: O18:H7; O25:NM: O26:NM: O26:H11: O26:H32; O38:H21: O39:H4: O45:H2: O50:H7: O55:H7: O55:H10: O82:H8: O84:H2: O91:NM: O91:H21: O103:H2: O111:NM; O111:H8: O111:H30: O111:H34: O113:H7: O113:H21: O114:H48: O115:H10: O117:H4: O118:H12: O118:H30: O121:NM: O121:H19: O125:NM: O125:H8: O126:NM: O126:H8: O128:NM: O128:H2: O128:H8: O128:H12: O128:H25: O145:NM: O125:H25: O146:H21: O153:H25: O157:NM: O163:H19: O165:NM: O165:19; and O165:H25
 - 24. The method of Claim 22 wherein said antitoxin comprises antitoxin directed against at least one *Escherichia coli* verotoxin.
- The method of Claim 22 wherein said antitoxin is cross-reactive with at least one *Escherichia coli* verotoxin.

26. The method of Claim 22 wherein said antitoxin is reactive against toxins produced by members of the genus *Shigella*.

- 27. The method of Claim 26, wherein said antitoxin is reactive against toxins produced by Shigella dysenteriae.
 - 28. A method for detecting Escherichia coli verotoxin in a sample comprising:
 - a) providing:

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- i) a sample:
- ii) an antitoxin raised against Escherichia coli verotoxin; and
- iii) a reporter reagent capable of binding said antitoxin: and
- b) adding said antitoxin to said sample so that said antitoxin binds to the Escherichia coli verotoxin in said sample.
- 15 29. The method of Claim 28, wherein said antitoxin is an avian antitoxin.
 - 30. The method of Claim 28, further comprising the steps of:
 - c) washing said unbound antitoxin from said sample:
 - d) adding said reporter reagent to said sample so that said reporter reagent binds to said bound antitoxin:
 - e) washing said unbound reporter reagent from said sample; and
 - f) detecting said reporter reagent bound to said antitoxin bound to the Escherichia coli verotoxin so that the verotoxin is detected.
- 25 31. The method of Claim 30 wherein said detecting is selected from the group consisting of enzyme immunoassay, radioimmunoassay, fluorescence immunoassay, flocculation, particle agglutination, and *in situ* chromogenic assay.
 - 32. The method of Claim 30 wherein said sample is a biological sample.
 - 33. The method of Claim 30 wherein said sample is an environmental sample.

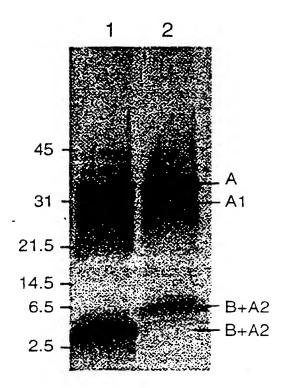
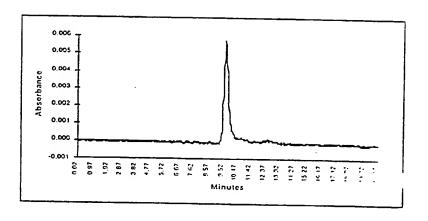


FIG. 1

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SUBSTITUTE SHEET (RULE 26)

Figure 2.

HPLC of rVT1



HPLC of rVT2

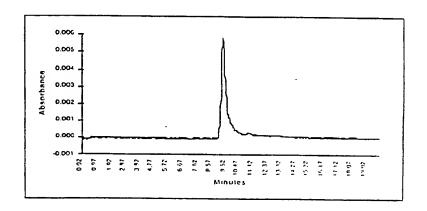


Figure 3. rVT1 and rVT2 Toxicity in Vero Cell Culture

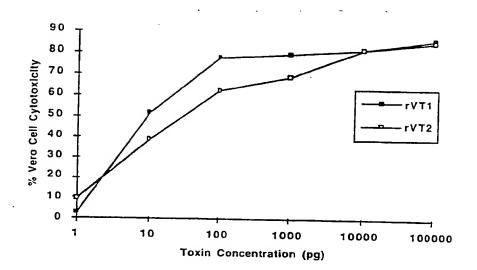


Figure 4.
EIA Reactivity of rVT1 and rVT2 Antibodies to rVT1

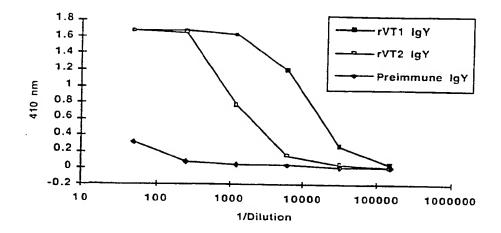
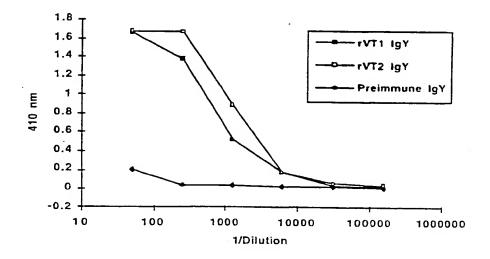


Figure 5.
EIA Reactivity of rVT1 and rVT2 Antibodies to rVT2



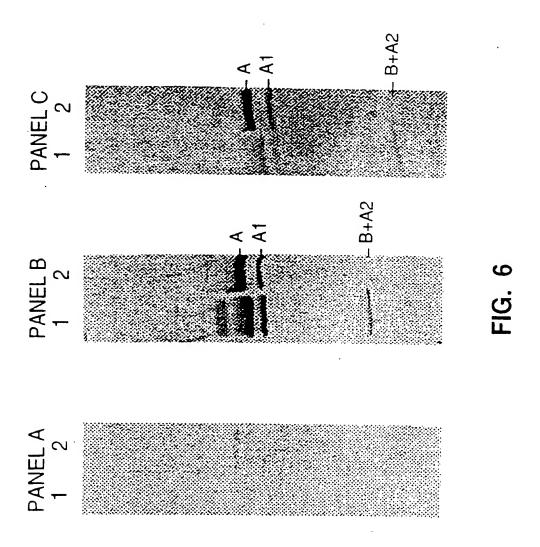


Figure 7.

Neutralization of rVT1 Cytotoxicity in Vero Cells

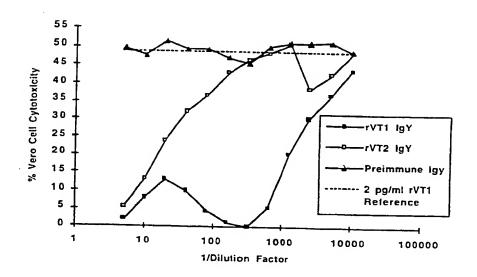
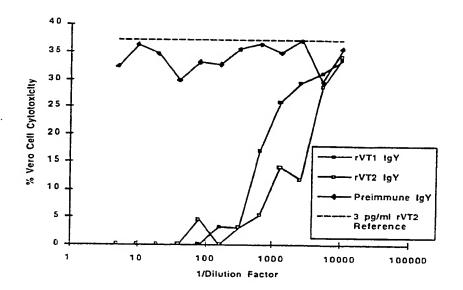


Figure 8.

Neutralization of rVT2 Cytotoxicity in Vero Cells



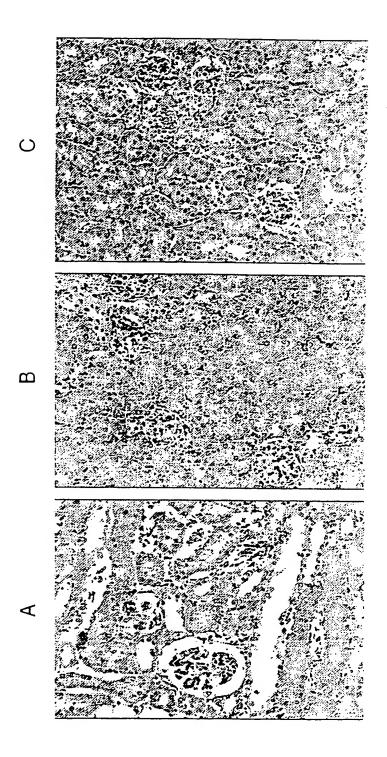
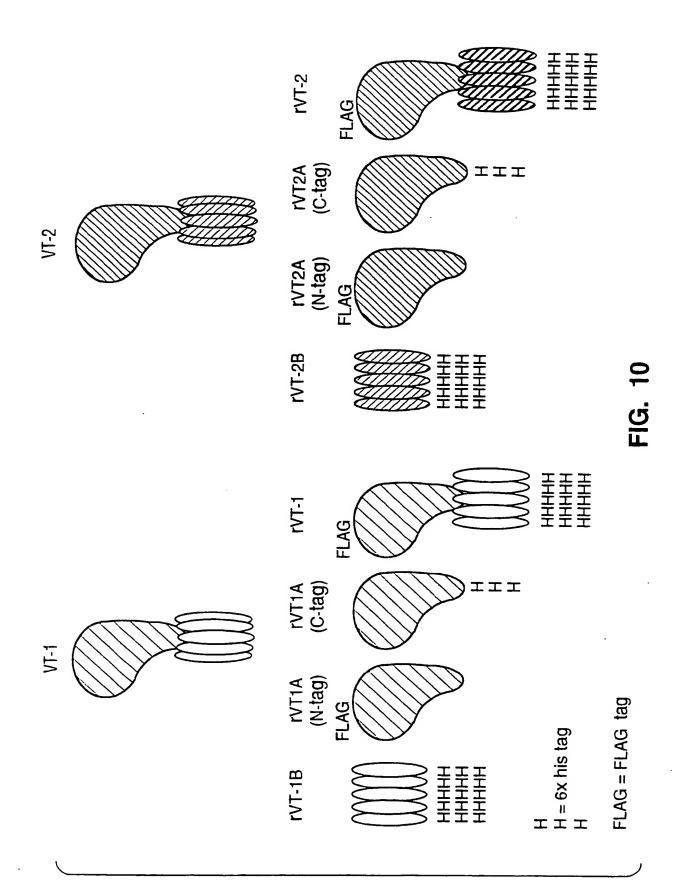


FIG. 9

1/



SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No. PCT/US96/04093

		PCT/US96/0	4093					
US CL	ASSIFICATION OF SUBJECT MATTER (A61K 39/00, 39/02; G01N 35/537 (Please See Extra Sheet.							
	to International Patent Classification (IPC) or to b LDS SEARCHED	oth national classification and IPC						
	documentation searched (classification system follo	Most by alamicania						
U.S. :								
Document	ation searched other than minimum documentation to	the extent that such documents are include	ed in the fields searched					
Electronic Please S	data base consulted during the international search See Extra Sheet.	(name of data base and, where practicable)	e, search terms used)					
C. DOC	CUMENTS CONSIDERED TO BE RELEVANT							
Category®	Citation of document, with indication, where	appropriate, of the relevant passages	Relevant to claim No.					
Y	BOYD et al. Serological Response Like Toxin 1 and Its Peptide Fra Subunit Is a Vaccine Candidate T Toxin. Infection and Immunity. M pages 750-757.	gments Indicate that the B	1-33					
Υ	US 5,326,559 A (MILLER) 05 Ju	lly 1994, columns 4-7.	1-33					
× Y	US 5,164,298 A (LINGWOOD e columns 10-13.	et al) 17 November 1992,	28, 30, 31, 32; 33 1-27 and 29					
Y	US 4,748,018 A (STOLLE et al) lines 25-55.	31 May 1988, column 4,	3, 21, 22, 29					
X Furth	er documents are fisted in the continuation of Box (e. See patent family annex.						
'A' dos	ceal categories of cited documents unrent defining the general state of the not which is not considered se of particular relevance.	The later document published after the inte- date and not in conflict with the applicate principle or theory underlying the inve-	limit is a cit of the country and a constant					
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Date of the a	Date of the actual completion of the international search Date of mailing of the international search report							
10 JULY 1996 2 7 AUG 1996								
Name and mailing address of the ISA/US Commissioner of Patems and Trademarks Authorized officer								
Box PCT	. D.C. 2023	RACHEL FREED						
Facsimile No	. (703) 305-3230	Telephone No. (703) 308-0196	Car					

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INTERNATIONAL SEARCH REPORT

International application No. PCT/US96/04093

	PC 1703	70,04075
C (Continue	tion). DOCUMENTS CONSIDERED TO BE RELEVANT	
Category**	Citation of document, with indication, where appropriate, of the relevant passage	es Relevant to claim No
'	US 4,550,019 A (POLSON) 29 October 1985, column 4, lines 68.	46- 3, 21, 22, 29
,	US 5,204,097 A (ARNON et al) 20 April 1993, column 2, line 1-16, column 3, lines 33-56 and column 5, lines 53-67.	es 2 and 14
	<u>-</u>	

INTERNATIONAL SEARCH REPORT

International application No. PCT/US96/04093

A. CLA	SSIFICATION	OF SUBJEC	T MATTER:
US CI			

424/134.1, 141.1, 150.1, 157.1, 164.1, 169.1, 192.1, 200.1, 236.1, 241.1, 801, 804, 809, 826; 435/7.37; 436/538, 542, 543-547

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS, MEDLINE, APS

search terms: verotoxin, verocytoxin, shiga, rvt1, rvt2, rslt1 or rslt2, vaccin? or treat?, recombinant

Form PCT/ISA/210 (extra sheet)(July 1992)*